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Polycomb Repressive Complex 2 Regulates miR-200b in Retinal Endothelial Cells: Possible Implications in Diabetic Retinopathy

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A thesis submitted in partial fulfillment of the requirements for the degree in Master of Science

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POLYCOMB REPRESSIVE COMPLEX 2 REGULATES miR-200b IN RETINAL
ENDOTHELIAL CELLS: POSSIBLE IMPLICATIONS IN DIABETIC
RETINOPATHY

(Thesis Format: Monograph)

by

Michael Anthony Ruiz

Graduate Program in Pathology

A thesis submitted in partial fulfillment for the degree of Master of Science

The School of Graduate and Postdoctoral Studies

The University of Western Ontario

London, Ontario, Canada

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Abstract

Glucose-induced augmented vascular endothelial growth factor (VEGF) production is a key event in diabetic retinopathy. We have previously demonstrated that downregulation of miR-200b increases VEGF, mediating structural and functional changes in the retina in diabetes. However, mechanisms regulating miR-200b in diabetes are not known. Histone methyltransferase complex, Polycomb Repressive Complex 2 (PRC2), has been shown to repress miRNAs in neoplastic process. We hypothesized that, in diabetes, PRC2 represses miR-200b through its histone H3 lysine-27 trimethylation mark. We show that human retinal microvascular endothelial cells exposed to high levels of glucose regulate miR-200b repression through histone methylation, and that inhibition of PRC2 increases miR-200b while reducing VEGF. Furthermore, retinal tissue from animal models of diabetes, showed increased expression of major PRC2 components, demonstrating *in vivo* relevance. This research established a repressive relationship between PRC2 and miR-200b, providing evidence of a novel mechanism of miRNA regulation through histone methylation.

Keywords: Polycomb Repressive Complex 2, microRNA-200b, vascular endothelial growth factor, retinal endothelial cells

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Dedication

I would like to dedicate this thesis to my family, friends and mentors who have helped me along this journey. My family, especially my mother and father, have encouraged my curiosity and love for science since I was a child. This support became even stronger when I wanted to try something no one in the family had done in pursuing a masters degree.

I am also lucky to be blessed with a handful of close friends who have been there to share both the joyful and challenging times. Whether we talked about the histone methylation and microRNAs, or the Blue Jays, my friends have been instrumental in keeping me grounded on this journey.

Finally, this work is dedicated to my mentors who have seen me grow over my time in Pathology. To be able to learn how to be a professional from people I truly admire has been a critical lesson in this whole process. Together, all of these people have allowed me to close a chapter in London and open a new one in Mississauga. This work is dedicated to you.

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List of Abbreviations

AGE	advanced glycation endproduct
ANRIL	antisense non-coding RNA in the INK4 locus
BMI	body mass index
ChIP	chromatin immunoprecipitation
DCCT	Diabetes Control and Complications Trial
DMSO	dimethyl sulfoxide
DZNep	3-Deazaneplanocin A
ECM	extracellular matrix
EDIC	Epidemiology of Diabetes Interventions and Complications
EED	embryo ectoderm development
ELISA	enzyme-linked immunosorbent assay
eNOS	endothelial nitric oxide synthase
ERCC	excision repair cross complimenting
ERK	extracellular signal-regulated kinase
ET-1	endothelin 1
EZH1	enhancer of the zeste homolog 1
EZH2	enhancer of the zeste homolog 2
FN	fibronectin
FOXO	forkhead box O
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
GLUT-1	glucose transporter 1
H3K4me3	trimethylation at lysine 4 on histone subunit 3

H3K27me3	trimethylation at lysine 27 on histone subunit 3
HDMEC	human dermal microvascular endothelial cells
HG	high glucose, 25mM D-glucose
HOTAIR	HOX antisense intergenic RNA
HRMEC	human retinal microvascular endothelial cells
HIF-1 α	hypoxia- inducible factor-1 alpha
IGF-2	insulin-like growth factor
JNK	jun-N-terminal kinase
KDM6A	lysine-specific Demethylase 6A
KDM6B	lysine-specific Demethylase 6B
lncRNA	long non-coding RNA
MAPK	p38 mitogen-activated protein kinase
miRNA	microRNA
MnSOD	managenese superoxide dismutase
NADPH	Nicotinamide adenine dinucleotide phosphate
NF κ B	nuclear factor kappa B
NG	normal glucose, 5mM D-glucose
OSM	osmotic control, 20mM L-glucose+5mM D-glucose
OXR1	oxidation resistance 1
PARP	poly(ADP-ribose) polymerase
PI3K	phosphatidylinositide 3-kinase
PKC	protein kinase C
Pol2	RNA polymerase 2

PRC2	polycomb repressive complex 2
qPCR	quantitative polymerase chain reaction
ROS	reactive oxygen species
RT-PCR	reverse transcription polymerase chain reaction
siRNAs	small interfering RNAs
STZ	streptozotocin
SUZ12	Suppressor of Zeste 12
UKPDS	United Kingdom Prospective Diabetes Study
UTR	untranslated region
VASH1	vasoinhibin-1
VEGF	vascular endothelial growth factor
WHO	World Health Organization

Chapter 1: Introduction¹

¹ Parts of this chapter have been adapted from the following publications: **1. Ruiz, M.A.** & Chakrabarti, S. MicroRNAs: The Underlying Mediators of Pathogenetic Processes in Vascular Complications of Diabetes. *Can. J. Diabetes*. **37**, 339-344 (2013). and **2. Feng, B., Ruiz, M.A.** & Chakrabarti, S. Oxidative-stress-induced epigenetic changes in chronic diabetic complications. *Can. J. Physiol. Pharmacol.* **91**, 213-220 (2013).

1.1 Diabetes Mellitus

Diabetes mellitus is a metabolic disorder characterized by impaired glucose utilization. It is defined as an absolute or relative deficiency of insulin, leading to hyperglycemia (1). There are two major types of diabetes, which are distinct in both their etiology and treatment strategy. Type 1 diabetes is also known as juvenile-onset diabetes because patients typically present with glucose utilization problems in their youth (2). The etiology of type 1 diabetes is believed to result from auto-immune destruction of the beta-cells of the pancreas, which function normally to secrete insulin in response to elevations in blood glucose levels. As a result, these patients have an absolute lack of insulin.

Supplementation of exogenous insulin is the major treatment strategy for these patients, and continual blood glucose monitoring and insulin administration with pumps have allowed these patients to live relatively normal lives. Conversely, patients with type 2 diabetes present at middle to late age, although this trend is changing as we see more type 2 diabetes in children and young adults.

Etiologically, type 2 diabetes is believed to result from lifestyle habits, such as diets high in carbohydrates and lack of physical exercise. Obesity is well linked to insulin resistance. As a result, the pancreas must secrete insulin at a much more rapid rate than in healthy patients (2,3). Eventually the beta-cells become exhausted, resulting in impaired insulin secretion in combination with insulin resistance. Medications may be necessary to treat type 2 diabetes. For example metformin is commonly prescribed to type 2 patients and functions to improve

insulin sensitivity. In patients who have severe type 2 diabetes with very difficult to control blood glucose levels, insulin supplementation may be required.

While management of blood glucose levels has improved, regardless of whether a patient has type 1 or type 2 diabetes they remain susceptible in the long term to hyperglycemia-induced complications (4,5,6). These include diabetic retinopathy, cardiomyopathy, nephropathy and others. These complications can reduce the quality of the life of patients with diabetes and can even lead to early death. Investigating how hyperglycemia causes signaling changes at a cellular level can lead to new treatment strategies to prevent these complications.

1.2 Epidemiology

Incidence of diabetes is a growing phenomenon in recent years and affects both the developed and undeveloped world. The World Health Organization (WHO) estimates that more than 347 million people worldwide have diabetes (7). In the United States, the incidence of diabetes in individuals older than 18 years old has increased by three fold since 1980 (8). Indeed, diabetes is no longer a disease of the young (type 1) or of the elderly (type 2). The incidence of both type 1 and type 2 diabetes has increased in the adolescent population and working age population, highlighting the growing problem of diabetes in society (9,10).

In Canada, the growth of diabetes is more modest than in the United States but is significant none-the-less. Nearly 2.85 million Canadians are estimated to be currently living with diabetes, either diagnosed or undiagnosed.

Since 1999, the prevalence of diabetes in Canada has increased by 70% (8).

Type 1 diabetes remains the most prevalent type of diabetes in youth, however type 2 incidence is growing. Canadians with diabetes are at 3 times greater risk for cardiovascular disease, 12 times greater risk for end-stage renal disease and 20 times greater risk for limb amputation compared to healthy individuals.

Overall, diabetics have two fold increased risk of mortality. Research into pathogenic signaling mechanisms involved in diabetes is necessary to develop new treatment strategies to maintain the quality of life of patients with diabetes, as well as to prevent diabetes-associated early death.

1.3 Diabetic Retinopathy and Pathogenic Signaling

Diabetic retinopathy is a microvascular complication of diabetes and a leading cause of vision loss (11). In early diabetic retinopathy, hyperglycemia induces the expression of vasoactive and inflammatory factors that increase retinal capillary permeability, causing macular edema, and contributing to pericyte and endothelial cell loss (4). Extracellular matrix (ECM) proteins also are increased, contributing to basement membrane thickening (12). As diabetic retinopathy progresses, microaneurysms develop and new vessels are formed. New vessels may lead to bleeding and tractional retinal detachment, leading to vision loss. Sustained hyperglycemia, elevated blood pressure and abnormal plasma lipids are all important risk factors in the progression of chronic diabetic complications including retinopathy. However large scale studies in both types of diabetes, such as the Diabetes Control and Complications Trial (DCCT), the

Epidemiology of Diabetes Interventions and Complications (EDIC) and the United Kingdom Prospective Diabetes Study (UKPDS), have shown that hyperglycemia is the main factor in the development of microvascular complications (13,14,15). Intensive glycemic control is important to delay the onset and progression of diabetic retinopathy, however this cannot always be achieved.

1.3.1 Intracellular signaling events

There are numerous signaling events and pathways that are involved in altering the expression of vasoactive factors like vascular endothelial growth factor (VEGF). Chronic, poorly controlled glucose first leads to signaling changes in endothelial cells of the eye. Endothelial cells are particularly susceptible to hyperglycemia-induced damage due to their constitutive expression of glucose transporter 1 (GLUT-1) (16,17,18). As a result, elevated glucose first causes signaling changes in these cells, which manifests in altered signals to other cell types in target organs.

A number of mechanisms are involved in hyperglycemia-induced damage. Increased intracellular glucose increases protein glycosylation and leads to production of advanced glycation end products (AGEs), which disrupt protein signaling and trafficking (19). Furthermore, excessive intracellular glucose becomes shunted down other metabolic pathways, such as the Polyol pathway. This metabolic pathway reduces nicotinamide adenine dinucleotide phosphate (NADPH) levels and impairs the reducing capability of the cell, resulting in increased reactive oxygen species (ROS) (20,21). ROS is particularly damaging

in diabetes where defective antioxidant activity (ie. MnSOD) exacerbates damage to the retina. ROS stimulate the generation of intracellular signals and activate pathways such as protein kinase C (PKC), c-Jun-N-terminal kinase (JNK), and p38 mitogen-activated protein kinase (MAPK). These pathways induce numerous functional changes; including endothelial dysfunction mediated through altered endothelial nitric oxide synthase (eNOS) activity and enhanced superoxide production (22,23).

Signaling cascades from metabolic changes converge in the nucleus as activation of various transcription factors. For example, forkhead box O (FOXO) and nuclear factor κ B (NF- κ B), which can mediate the effects of ROS through regulation of gene transcription of inflammatory and antioxidant genes (24). Oxidative stress also causes DNA damage and together activates DNA repair enzymes, transcription factors, and transcription co-activators in endothelial cells. The activated nucleotide excision repair enzymes include excision repair cross complementing 1 (ERCC1), ERCC4 (also known as Xeroderma Pigmentosum complementation group F) and poly(ADP-ribose) polymerase (PARP) (25). In parallel, there is augmented production of histone acetyl transferase p300 and alterations of histone deacetylases including the class III deacetylases, the sirtuins (26,27,28). Abnormal chromatin modifications, such as acetylation, methylation, phosphorylation, sumoylation, and ubiquitination, occur at specific residues in the N-terminal tails of histones and further modulate gene expression (29). Altogether, these changes in the nucleus alter the expression of various

genes associated with diabetic complications, which further mediate the organ's response to hyperglycemic conditions.

1.3.2 Transcriptional changes and extracellular consequences

The functional consequence of the altered signaling events in diabetes is increased transcription of multiple vasoactive factors and ECM proteins, which are involved in the development and progression of chronic diabetic complications. Inflammatory and angiogenic cytokines elevated in diabetes signal to increase permeability and proliferation in the retina. These mediators include vascular endothelial growth factor (VEGF), endothelin (ET-1), interleukins (ILs) and other factors (30,31,32). Accumulation of ECM proteins, such as fibronectin (FN) or collagen, thickens the basement membrane and further contributes to vascular dysfunction and increased ocular permeability (12). As a result, vessel integrity is compromised and there is greater risk of microaneurysm and hemorrhage, which occur commonly in diabetic retinopathy. Over time the altered microenvironment induced by hyperglycemia changes and deteriorates the structure of the retina, leading to decreased function and vision loss.

1.3.3 Current treatment strategies

Maintaining tight glycemic control with medications and lifestyle changes has been the major therapeutic strategy and has been shown to prevent or slow down the development of diabetic retinopathy (33). However, some patients, still experience elevated glucose levels and thus remain susceptible to diabetic

retinopathy (4). Research into key pathogenic targets that mediate diabetic retinopathy has uncovered an important role of VEGF. VEGF is a growth factor that is elevated in the eye in diabetes, which has been shown at both mRNA and protein levels (34,35,36,37). VEGF causes increased vessel permeability in the early stages of diabetic retinopathy, and mediates endothelial cell migration and neovascularization in the late stages of diabetic retinopathy. Targeted therapy to inhibit VEGF has been a leading strategy for years in the treatment of diabetic retinopathy (38,39).

Targeted inhibitory therapy against VEGF receptor using a monoclonal antibody has also been shown to be effective in preventing microaneurysm formation and improving visual acuity in patients with diabetic retinopathy (38,39,40,41). While VEGF-antibody has shown efficacy in both of these aspects, pharmacological therapy is not effective for all patients. For example, 40% of patients show no improvements in visual acuity following VEGF-antibody treatment (41). It is also invasive, as these patients must have eye injections monthly for one to three years. Furthermore, in Ontario the current cost of VEGF-antibody treatment is over \$1,500 per injection, which is not funded by the provincial drug plans (42). As a result, this treatment carries a high cost to both the health care system and the patient. With diabetes becoming an epidemic across the developed and underdeveloped world, research into additional therapies is needed to improve our understanding of the pathogenic mechanisms of diabetic retinopathy, as well as to deliver a more efficacious treatment to more people.

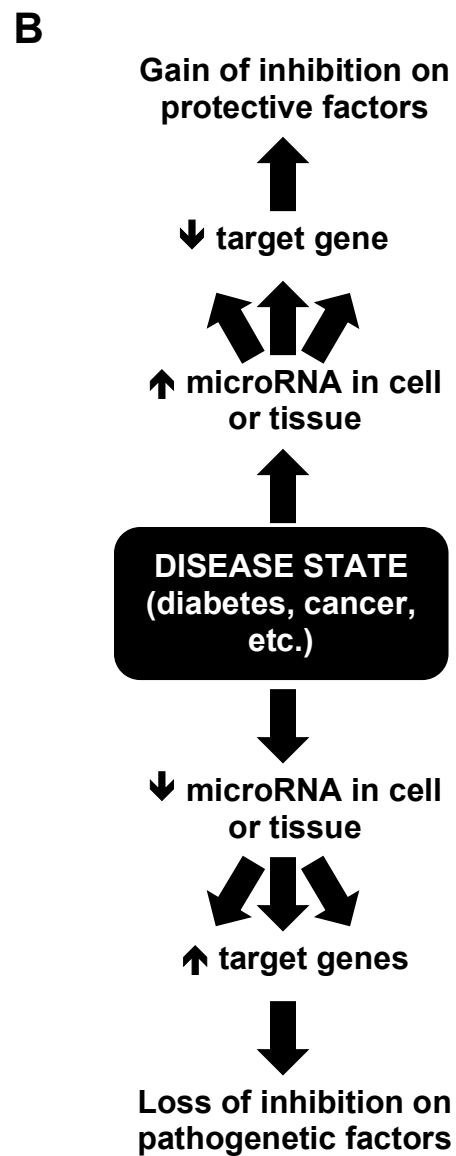
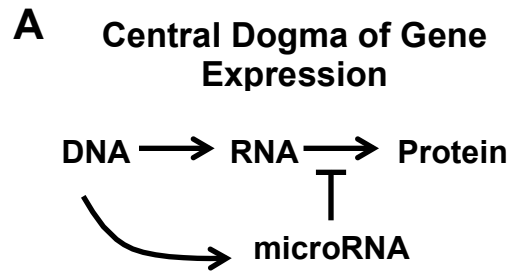
1.4 microRNAs

Recent research has uncovered an underlying layer of microRNA (miRNA) involvement in most biologic processes and several diseases, such as cancer or diabetes. miRNAs, a group of noncoding RNAs, are well-studied epigenetic elements and have shown important interactions in many pathogenetic processes. miRNAs are post-transcriptional regulators of gene expression. They are produced initially by RNA Polymerase 2 (Pol2) as immature transcripts and are processed into shorter, mature miRNAs (43,44). Mature miRNAs target messenger RNAs (mRNAs) for inhibition through specific binding at the 3' untranslated region (UTR), triggering mRNA degradation or halting translation depending on complementarity (45). Unlike miRNA, other noncoding RNAs have more complicated functions in chromosomal modifications, gene transcription and translation (46). miRNAs target many genes for inhibition, and databases have been developed to predict these interactions (47,48,49). Currently, more than 700 miRNAs have been identified in the human genome and it is estimated that approximately 30% of the human genome is regulated by miRNAs (50). From an evolutionary perspective, miRNAs represent another level of control over cellular events by tightly regulating gene expression (Figure 1.1A). Therefore, aberrant miRNA expression in disease processes can disrupt normal cell physiology and mediate pathogenetic processes (51,52). Altered miRNA expression generally has 2 possibilities: increased miRNA expression inhibits specific mRNA expression, whereas decreased miRNA expression enhances specific mRNA expression (Figure 1.1B). In diabetes, altered metabolic events

Figure 1.1

Functions and implications of altered microRNA expression in disease states.

(A) At the post-transcriptional level, miRNAs function to regulation gene expression by targeting mRNAs for degradation or inhibition. (B) Increased miRNA results in increased inhibition of target genes, often resulting in reduced protective factors. Decreased miRNA results in decreased inhibition of target genes, mediating pathogenetic signaling. [Figure adapted from reference #95]



lead to alterations of gene transcription. Such changes may also lead to changes in miRNA expression as both are under similar regulation (51).

miRNAs have been silently involved in the pathogenesis of many diseases. It is only in recent years that the importance of these background players is beginning to be understood in chronic diabetic complications. Understanding epigenetic elements, such as miRNA, can yield new therapeutic strategies.

1.5 microRNAs in diabetic retinopathy

1.5.1 miRNAs involved with vasoactive factors and matrix protein accumulation

In diabetic retinopathy, several miRNA have been shown to be involved. Major investigations have been performed with miR-200b and miR-146a. In this section, several miRNAs related to diabetic retinopathy will be discussed, as well as the potential therapeutic efficacy of one promising miRNA, miR-200b.

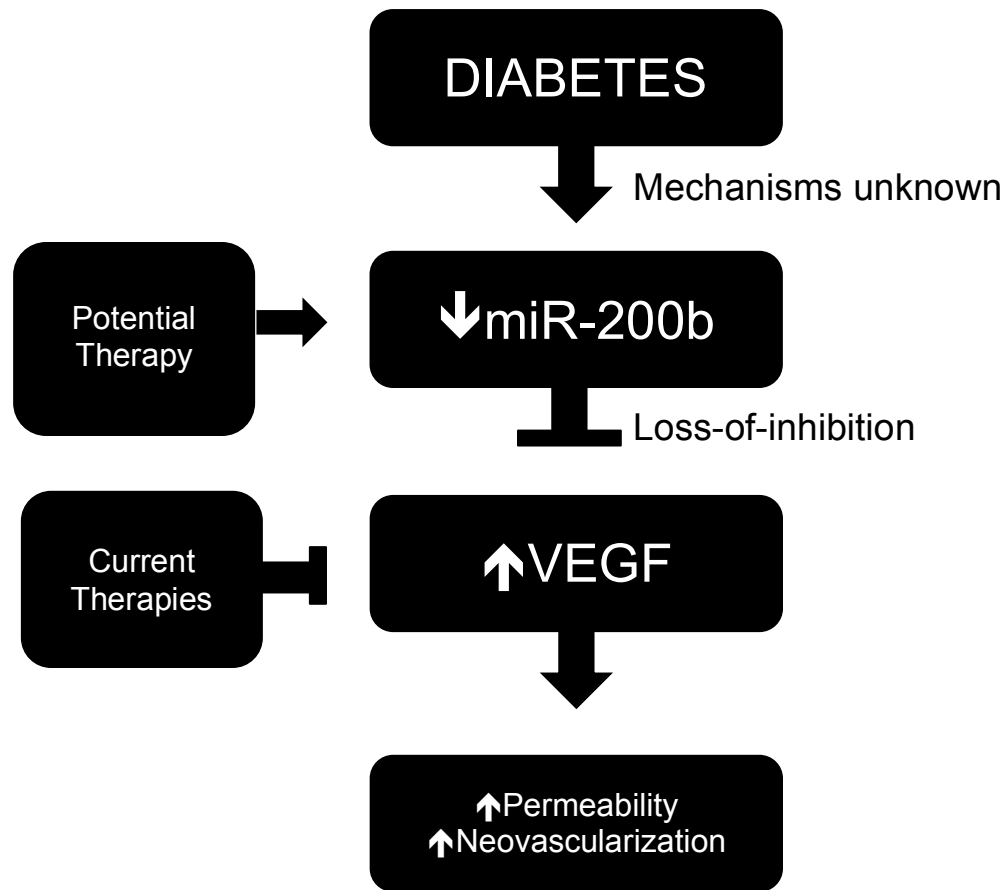
Increased retinal capillary permeability and other changes are mediated by increased production of specific factors, including VEGF. One miRNA, miR-200b, has been shown to regulate VEGF (53). miR-200b is produced from cluster of human chromosome 1, along with related miRNAs miR-200a and miR-429. Our laboratory has shown decreased miR-200b in bovine retinal endothelial cells exposed to high levels of glucose, as well as in retinal tissue of streptozotocin-induced (STZ) diabetic rats at 1 month following diabetes induction. Due to loss

of negative regulation, VEGF expression and retinal permeability were enhanced. Intravitreal injections of miR-200b were protective by reducing VEGF as well as vessel permeability (53). Furthermore, miR-200b levels were decreased in human retinal tissue from diabetic patients compared to non-diabetic patients, further supporting that loss of miR-200b occurs in diabetic retinopathy and that restoring miR-200b may be therapeutically useful (Figure 1.2). Interestingly, another study in a genetic model of type 1 diabetes (BL/65-Ins2 Akita) found increased miR-200b expression relative to B6 control mice (54). In this model, increased miR-200b inhibited oxidation resistance 1 (Oxr1), a protective gene involved in resistance to oxidative stress. These studies differed in diabetic models (STZ vs. Akita), time points (1 month vs. 8 months), cell types cultured and studied (endothelial vs. Mueller cells) and as well as *in vitro* treatment conditions, which may have accounted for the differences in miR-200b expression relative to the non-diabetic controls. Nevertheless, it is possible that altered expression of miR-200b in either direction could have consequences.

Matrix protein accumulation is another important feature contributing to structural abnormalities and eventual neovascularization (12). miR-146a regulates FN levels and is downregulated in the retina of the STZ rat model at 1 month of diabetes (55). Furthermore, intra-ocular injection of miR-146a mimic reduced FN accumulation. miR-320 is downregulated in diabetic retinopathy and regulates extracellular signal related kinases 1 and 2 (ERK1/2; part of MAPK pathway) and activation of VEGF, ET-1 as well as FN. Restoring miR-320 may be protective in diabetic retinopathy by targeting ERK1/2 signalling (56).

Figure 1.2

Relationship between diabetes, microRNA-200b (miR-200b) and vascular endothelial growth factor (VEGF). Previous work in our laboratory has demonstrated that miR-200b is downregulated in diabetes, leading to increased VEGF expression and increased permeability (44). Intraocular injection of miR-200b mimic was found to be effective in reducing VEGF expression and vascular permeability, demonstrating miR-200b as a potential therapeutic agent. The understanding into why miR-200b becomes repressed is limited and is the focus of the current study.



Likewise, miR-126 is protective by inhibiting vasoactive factor expression, and loss of miR-126 resulted in increased VEGF levels, insulin-like growth factor (IGF)-2 and hypoxia-inducible factor-1 alpha (HIF-1 α) (57). These factors play a major role in late-stage proliferative diabetic retinopathy by further mediating permeability changes and ECM protein accumulation, which eventually contribute to neovascularization and vision loss (4).

1.5.2 Therapeutic potential

Overall, miRNAs are important mediators of vascular permeability, matrix protein accumulation and neovascularization, which together can contribute to vision loss over time in diabetic patients. Of all the miRNAs described, miR-200b may have the greatest therapeutic potential for its regulation on VEGF. Administration of exogenous miR-200b may be efficacious in the treatment of the microvascular events of diabetic retinopathy, however there are several limitations. Most obviously, miRNAs are endogenous small interfering RNAs (siRNAs), and the current use of siRNAs as a therapy is limited only to experimental purposes (58). While siRNAs and miRNAs offer compelling therapeutic targets, little is understood about their safety, and the multitude of potential interactions could be a double-edged sword in disease management (59). Furthermore, although endogenous delivery mechanisms are being investigated, miRNAs have short half-lives in circulation and thus would have to be injected directly into the eye to treat a disease such as diabetic retinopathy,

offering no logistical advantage to current therapies (60). Instead, identifying a large molecule (ie. protein) which regulates these miRNAs may be a more attractive therapeutic strategy.

Understanding into why miRNAs, like miR-200b, become dysregulated in diabetes is important to further our understanding of the disease, as well as identify molecular targets that may be important from a therapeutic perspective. Additional research in this topic is necessary, and current research suggests an epigenetic link between microRNA regulation and histone methylation.

1.6 An epigenetic connection: histone methylation

Histone modification is another emerging theme in the field of epigenetics and is involved in the coordination of gene expression. Amino acid residues within histone subunit molecules become modified in a variety of ways (ie. acetylation, methylation, phosphorylation etc.), by chromatin modifying enzymes, which changes the overall accessibility of chromatin and affects how transcription factors can activate/repress genes in the modified area (61). For example, acetylation can be thought of as an “activating mark,” as areas of the genome in which histones are highly acetylated show high gene expression (62,63). The addition of an acetyl group is thought to make the chromatin more positively charged and thus more accessible for transcriptional machinery. Methylation is another histone modification process involved in gene expression but is more complicated and not as well understood as acetylation (64).

There are several key principles to keep in mind when discussing histone methylation. First, the amino acid which is methylated is important. Depending on the amino acid residue, the result can be activation or repression of gene expression in that area of the genome (65,66). For example, tri-methylation at lysine 4 on histone subunit 3 (H3K4me3) is typically activating, leading to recruitment of transcription factors and increased expression of genes. Conversely, tri-methylation at lysine 27 on histone subunit 3 (H3K27me3) is typically repressing, leading to closed chromatin.

Second, existing methyl-marks can be recognized. Chromatin-modifying enzymes are able to recognize existing marks on histones with high specificity (67). For example, the recruitment of Polycomb Repressive Complex 2 (PRC2), a repressing histone methyltransferase complex, to a region of the genome is inhibited by existing H3K4, H3K36 methylation, H3K27 acetylation or H3K28 phosphorylation (68). Past histone modification can therefore affect future genomic interaction between other modifying enzymes.

Third, methyl-marks can be removed. Histone methylation is a reversible and dynamic process and there are many histone demethylases for each type of methylation (69). For example, Lysine-specific Demethylase 6A (KDM6A) and 6B (KDM6B) catalyze the demethylation of H3K27me3 and thus oppose or balance the activity of PRC2 (70). This reversibility of histone methylation adds to the complexity of interactions but allows for a more attractive therapeutic strategy.

Finally, methyltransferases often complex with adaptor proteins or long non-coding RNA (lncRNA). Methyltransferases achieve specificity to regions of

the genome by associating with adaptor proteins and lncRNA. For example, Suppressor of Zeste 12 (SUZ12) and Embryo Ectoderm Development (EED) are adaptor proteins that interact with Enhancer of the Zeste Homolog 2 (EZH2) to form PRC2, the major methyltransferase complex for H3K27 methylation. EED and SUZ12 allow for PRC2 recruitment to specific parts of the genome, which can be direct through complex protein structures or indirect through lncRNA (68,71). lncRNA are able to guide chromatin-modifying enzymes to specific genes through their polar structure; in which one end integrates within the gene and the free end recruits the enzyme (46). Similarly, lncRNA can act as molecular scaffolds to increase interactions between transcription factors and other chromatin modification enzymes.

Research into the expression and activity of histone methyltransferases is booming in the cancer field and is becoming attractive in the diabetes field due to the recently described phenomena of “metabolic memory.” Changes in gene expression and resultant vessel functionality due to hyperglycemia have been observed to persist long after returning to a normoglycemic state (72). So far only H3K4 and H3K9 methylation have been examined in cell culture studies and animal models of diabetes. However, evidence suggests that methylation is indeed a therapeutically targetable mechanism in chronic diabetic complications (73,74,75,76).

1.7 Polycomb Repressive Complex 2

One particular type of methylation, H3K27me₃, has been linked to miRNA regulation in several investigations. PRC2 is a repressing histone methyltransferase complex that has been linked to negatively regulating many genes, including miRNAs. In this section, the structure and function of PRC2 will be discussed, as well as potential regulatory relationships that may be of interest in diabetic retinopathy.

1.7.1 Structure and Function of PRC2

PRC2 is a multimeric complex that catalyzes trimethylation of lysine 27 on histone H3 (H3K27me₃). This is a repressing type of methylation which leads to closed and inaccessible chromatin (77,78). Thus, PRC2 is believed to be a negative regulator of gene expression. The complex is composed of a methyltransferase portion, adaptor proteins, as well as lncRNA, which function in unison to regulate gene expression. Arguably the most important subunit is EZH2, which catalyzes the H3K27 trimethylation reaction (78). EZH2 has been extensively studied in human cancers and is responsible for alterations in tumour vasculature and invasiveness (79,80). Two other subunits of particular importance are EED and SUZ12. Unlike EZH2, EED and SUZ12 do not have direct methyltransferase catalytic function but instead are responsible for recruitment of EZH2 to different promoter regions (81,82). Specificity to different regions of the genome is mediated by direct protein-DNA interactions, as well as by recruitment through lncRNA, such as ANRIL or HOTAIR (83). These lncRNA

function as molecular flags to direct PRC2 to specific genome areas. They may also act as scaffolds to bring together chromatin remodeling complexes. Thus, the methyltransferase function of PRC2 is mediated by EZH2 while its specificity is mediated by EED and SUZ12, as well as lncRNA.

Most of the functional importance of PRC2 has come from research in the cancer field. In a variety of cancers, PRC2 components such as EZH2 and SUZ12 are over expressed, leading to increased H3K27me3 at a variety of gene promoters. The functional result is silencing of expression in that area, resulting in increased angiogenesis and enhanced metastasis (84). Furthermore, increased expression of this complex has been shown to be induced by VEGF. PRC2 is known to negatively regulate vasoinhibin-1 (VASH1), an anti-angiogenic factor, and can thus further enhance the angiogenic action of VEGF (79,85). Finally, PRC2 has been implicated in regulating cell-cell adhesion and inflammatory genes in endothelial cells (86).

1.7.2 PRC2 and microRNA regulation

PRC2, and specifically the EZH2 and SUZ12 subunits, have been shown to regulate several microRNAs in the context of neoplasia. Interestingly, there is an overlap of several microRNAs, which are repressed both in diabetic complications and cancer (87). For example, miR-200b is important in the context of diabetic retinopathy for its regulation on VEGF, which mediates increased permeability in the eye and eventual neovascularization as the disease progresses (53). miR-200b is also implicated in several cancers (88). In cancer,

miR-200b is known as tumour suppressor gene that becomes downregulated or mutated in cancer (88). This loss of inhibition leads to increased expression of target genes, which mediates increased tumour invasiveness. In human fibroblasts, increased H3K27me3 was found at the promoter regions of the miR-200 gene cluster with correlating decreased expression relative to epithelial cells, illustrating a mechanism for how miR-200b becomes repressed (89).

1.8 Rationale

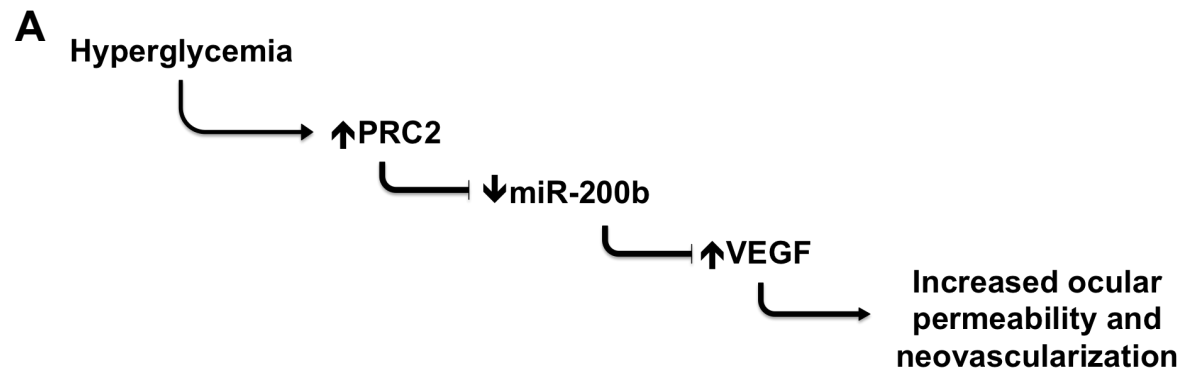
While there is limited understanding as to why microRNAs like miR-200b become repressed in diabetic complications, based on the above discussion, it is possible that the underlying regulation of miR-200b may involve PRC2 (Figure 1.3). Altogether, the importance of miR-200b in diabetic retinopathy, with possible links of PRC2 to miR-200b, provides clear rationale for investigating this complex in the context of diabetic retinopathy. Involvement of PRC2 in diabetic retinopathy remains elusive, as well as links to VEGF and angiogenesis, providing additional motivation for the current study.

1.9 Hypothesis

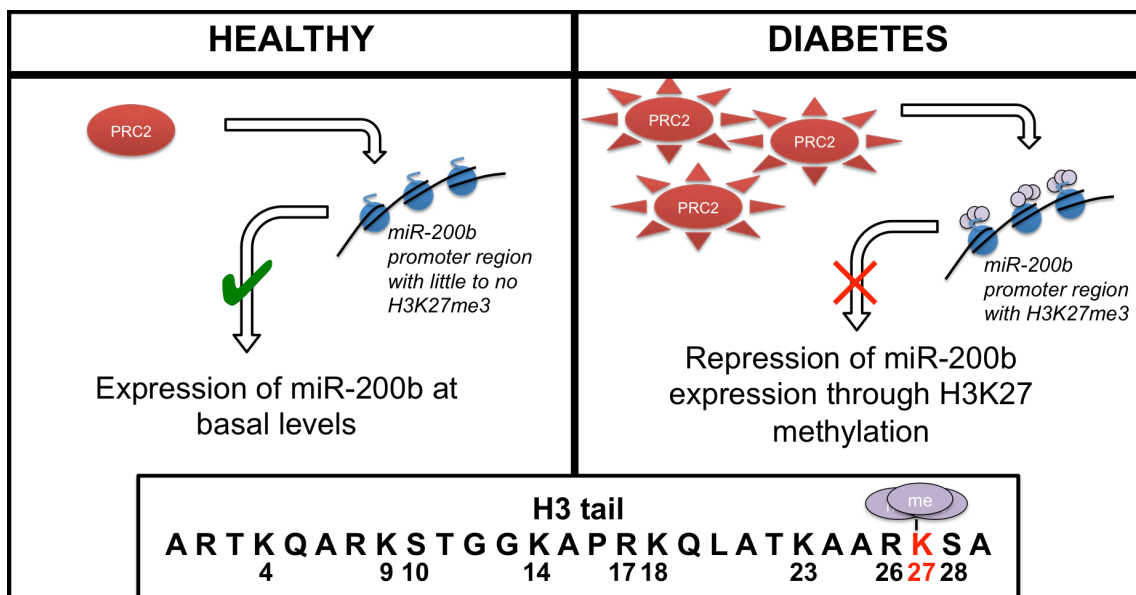
We hypothesize that glucose induces increased PRC2 expression and activity, which causes alteration of miR-200b levels. Such changes may play a role in the development of diabetic retinopathy.

Figure 1.3

Schematic showing our working hypothesis. (A) Glucose-induced increased PRC2 expression and activity causes alteration of miR-200b levels. Such changes may play a role in the development of diabetic retinopathy. (B) This relationship is hypothesized to be regulated at the level of genome by PRC2-mediated repression through H3K27me3 at the miR-200b promoter region.



B



1.10 Specific Aims

To test our hypothesis, we have devised 4 objectives:

1. To investigate the effect of glucose on VEGF levels in human retinal microvascular endothelial cells.
2. To investigate the effect of glucose on PRC2 levels and activity in human retinal microvascular endothelial cells.
3. To investigate whether PRC2 components are altered in retinal tissues of diabetic animals.
4. To investigate the regulatory relationship between PRC2 and miR-200b.

Chapter 2: Materials and Methods

2.1 In Vitro Studies

2.1.1 Human Retinal Microvascular Endothelial Cells

To investigate PRC2 and miR-200b regulation in the context of diabetic retinopathy, the major cell type used for investigation was the human retinal microvascular endothelial cell (HRMECs, Olaf Pharmaceuticals, Worcester, MA).

HRMECs were grown and passaged in tissue culture flasks in Endothelial Basal Medium 2 (EBM-2; Lonza, Walkersville, MD) containing 5mM D-glucose, 10% fetal bovine serum (FBS; Sigma-Aldrich, Oakville, ON) and all provided growth factors and antibiotics (EBM-2 Single Quots). No additional antibiotic was added to the medium. HRMECs were incubated at 37°C with 5% CO₂ and used between passages 3 and 8 to minimize variability. Prior to experimentation, HRMECs were seeded on the appropriate dish based on the assay. At 80-90% confluency, HRMECs were serum starved overnight by removing the growth medium and replacing it with EBM-2 containing 0% FBS, no growth factors or antibiotics. Serum starvation is important to remove the confounding variables (growth factors) in serum. Following starvation, cells were treated with additional D-glucose (Sigma-Aldrich, Oakville, ON) to represent high glucose levels (HG, 25mM) with normal glucose controls (NG, 5mM). Osmotic controls were also used where necessary (20mM L-glucose + 5mM D-glucose). All experiments were performed with 6 replicates unless noted otherwise.

2.1.2 Human Dermal Microvascular Endothelial Cells

While HRMECs are the focus of most of the work in this study, other endothelial cell types were investigated to elucidate whether genes associated with PRC2 might be specific to retinal endothelial cells. Furthermore, human dermal microvascular endothelial cells (HDMECs) have been used to model diabetic vascular complications by our laboratory and others.

HDMECs isolated from non-diabetic individuals (Lonza) and type 1 and type 2 diabetic individuals (Lonza) were cultured identically to HRMECs. HDMECs from non-diabetic individuals were isolated from adult foreskin and were pooled together from multiple donors. HDMECs of type 1 diabetes origin (type 1) were isolated from a 62-year-old female type 1 diabetic patient with a body-mass index (BMI) of 22. HDMECs of type 2 diabetes origin (type 2) were isolated from a 73-year-old female type 2 diabetic patient with a BMI of 29.

2.2 In Vivo Studies

Male Sprague–Dawley rats (175 g, 6 weeks old) were obtained (Charles River, Wilmington, MA, USA). Diabetes was induced by a single intraperitoneal injection of streptozotocin (STZ, 65mg/kg, in citrate buffer 5.6pH), with control rats receiving an identical volume of citrate buffer. Rats were monitored for changes in body weight and blood glucose. After 4 weeks of diabetes duration, rats were sacrificed, retinal tissue was collected and RNA was extracted as described below. All animal experimentation was carried out in accordance to Western University and Animal Care and Veterinary Services Guidelines.

Male C57BL/6 mice (23-36gm, were administered three doses STZ (50mg/kg) to induce diabetes, with control animals receiving an identical volume of citrate buffer. Animals were monitored for changes in body weight and blood glucose. After 8 weeks of diabetes duration, mice were sacrificed and retinal tissue was collected.

2.3 RNA Extraction and Real Time RT-PCR

mRNA was extracted using techniques already established in our lab. Briefly, cells were collected in of TRIZOL™ reagent (Invitrogen, Burlington, ON). 0.2mL of chloroform was added to the tube containing TRIZOL™, vortexed vigorously. The samples were centrifuged at $15,000 \times g$ for 15 minutes at 4°C and the aqueous layer was carefully recovered in a fresh tube. An equal volume of isopropanol was added to the aqueous layer. The mixture was vortexed briefly, and centrifuged at $15,000 \times g$ for 15 minutes at 4°C. The pellet was washed with 75% ethanol, dried, resuspended in DEPC water and quantified with spectrophotometry (260nm; Gene Quant, Pharmacia Biotech, USA).

To generate cDNA, 2µg of total RNA was used with the High Capacity Reverse Transcription Kit (Applied Biosystems, Foster City, CA) and random hexamer primers. Quantitative Real time RT-PCR was performed using a Roche LightCycler 96 (Roche, Laval, QC) and SYBR Green detection (Clontech, Mountain View, CA). The reaction mixture (total volume 20µL) consisted of the following components: 10µL of SYBR Advantage qPCR Premix, 1µL of each 10µM forward and reverse primer, 1µL cDNA sample and 7µL nuclease free

water. Primers for VEGF, EZH2, EED, SUZ12, KDM6A, and KDM6B are shown in Table 2.1 (Qiagen, Germantown, MD and Sigma-Aldrich). Normalization was performed to β -actin to account for differences in reverse transcription efficiencies of cDNA.

2.4 miRNA Extraction and Real Time RT-PCR

To isolate miRNA, mirVana microRNA Isolation Kit (Ambion, Austin, TX) was used. Cells were collected using and centrifuged at $200 \times g$ for 4 minutes. The cell pellet was washed, transferred to a centrifuge tube and spun at $2600 \times g$ rpm for 4 minutes. The pellet was resuspended in 500 μ L of lysis buffer. 50 μ L of miRNA Homogenate Additive was added to the lysate and the samples were left on ice for 10 minutes. Five hundred μ L of Acid-Phenol:Chloroform (1:1) was added to the lysate and the mixture was vortexed. The mixture was centrifuged at $15,000 \times g$ for 10 minutes at 4°C to separate the organic and aqueous layers. The aqueous layer was carefully removed and transferred to a fresh tube. Six hundred twenty five μ L of 100% ethanol was added to the aqueous layer and the mixture was vortexed briefly. The mixture was filtered through a filter column in 600 μ L batches, each time discarding the filtered solution following centrifugation. Sixty μ L of preheated (95°C) Elution Solution was added to the filter column and the filter was centrifuged for 1 minute in a fresh tube. The RNA concentration was quantified using spectrophotometry (260nm).

To generate cDNA, 100ng of total RNA was used with Reverse transcription was performed using the High Capacity Reverse Transcription Kit.

Table 2.1 Oligonucleotide sequences RT-PCR

Genes	Primer Sequences (5' → 3')
β-actin (human/rat/mouse)	CCTCTATGCCAACACAGTGC CATCGTACTCCTGCTTGCTG
VEGF (human)	GAAC TTTCTGCTGTCTTGGG CTTCGTGATGATTCTGCCCT
EZH2 (human)	QIAGEN (QT00054614)
EED (human)	QIAGEN (QT00074627)
SUZ12 (human)	TACGGCTCCTATTGCCAAAC TGCTTCAGTTTGTTGCCTTG
KDM6A (human)	QIAGEN (QT00094654)
KDM6B (human)	QIAGEN (QT00098742)
VASH1 (human)	GTTCCCTCCGAAACTGAGAC ACAAAGCACCCCCATCTAAC
EZH2 (rat)	GCACACTGCAGAAAGATCCA AGGTAGCACGGACACTGCTT
EZH2 (mouse)	AGACGTCCAGCTCCTCTGAA CATCCTCAGTGGGAACAGGT
EED (rat/mouse)	CTGGCAAAATGGAGGATGAT GGTCAGTGTTGTGCATTTGG
SUZ12 (rat/mouse)	GTCTCAGGGGTTCCAAAACA ACACTGCCTGTTCCAAATCC

Specific primers are necessary to analyze mature miRNAs due to their short size. The RT-primer extends the template to create a suitable amplicon length for PCR analysis. TaqMan miRNA Assays (Applied Biosystems) were used as primers for Real Time RT-PCR (Table 2.2). miR-200b expression was quantified using this method and normalization was performed to house keeping gene U6.

2.5 Chromatin Immunoprecipitation (ChIP) qPCR Analysis

To elucidate changes in chromatin modifications associated with high glucose, ChIP-qPCR was performed. This technique allows pull down of specific protein targets and confirms genome association by performing qPCR with primers against select genomic regions. Chromatin Immunoprecipitation (ChIP) Assay Kit (Milipore, Temecula, CA) was used according to manufacturer's instructions. Following 24 hours of glucose exposure, the cells were fixed using formalin. The plates were incubated at 37°C for 10 minutes and were immediately removed and washed twice with D-PBS. Five mL of D-PBS containing protease inhibitors was added to the plate and cells were carefully suspended using a cell lifter. The D-PBS mixture was transferred to a 15mL tube. An additional 5mL of D-PBS containing protease inhibitors was added to the plate to remove any remaining cells. The cells were centrifuged at $200 \times g$ for 4 minutes, and the pellet was washed once with D-PBS, transferred to a 1.7mL tube and recentrifuged to produce a pellet. The pellet was resuspended in 200 μ L of SDS lysis buffer containing protease inhibitors (Roche) and incubated on ice for 30 minutes, with brief vortexing at 10 minute intervals. The lysate was

Table 2.2 RT-PCR TaqMan miRNA Probe Sequences

Genes	Probe Stem-Loop Sequence (5' → 3')
mature miR-200b	CCAGCUCGGGCAGCCGUGGCCAUCUUACUGGGCAG CAUUGGAUGGAGUCAGGUCUCUAAUACUGCCUGGU AAUGAUGACGGCGGAGCCCUGCACG
U6	GTGCTCGCTTCGGCAGCACATATACTAAAATTGGAAC GATACAGAGAAGATTAGCATGGCCCCTGCGCAAGGAT GACACGCAAATTCGTGAAGCGTTCCATATTTT

sonicated on ice three times in 30 second intervals with a KONTES Micro-Ultrasonic Cell Disruptor (power of 6 at a tuning of 4) to attain DNA fragments in the 200-1000bp range, appropriate for immunoprecipitation. The lysate was centrifuged at $18,000 \times g$ for 10 minutes to remove any insoluble cellular components and the supernatant was transferred to a fresh tube. The supernatant was precleared using Salmon Slurry Agarose for 30 minutes at 4°C with rotation. The mixture was briefly centrifuged for 1 minute at $100 \times g$ and was transferred to a fresh tube. Eighty μL (5%) of each sample was transferred to a fresh tube and stored at -80°C for an input measurement. For each immunoprecipitation reaction, 1.6mL of each sample was used and extra sample discarded. Four μL of antibody (1:400 dilution) was used for each immunoprecipitation reaction. Antibody-chromatin mixture was incubated overnight at 4°C with rotation.

Following 14 hours of rotation, 60 μL of Salmon Slurry Agarose was added to each sample and the mixture was incubated for 1 hour at 4°C with rotation. The mixture was then centrifuged at $100 \times g$ for 2 minutes and the agarose pellet was washed and re-centrifuged three times at 4°C (1. Low Salt Solution, 2. High Salt Solution, 3. Li-Cl Solution) and twice at room temperature (both 8.0 pH EDTA). Each wash was performed for 5 minutes with rotation. Following the final wash and centrifuge, precipitated DNA was released using 250 μL of freshly prepared elution solution (1% SDS, 1M NaHCO_3) rotating for 15 minutes at room temperature. This elution was repeated to assure effectiveness, for a 500 μL eluate final volume. Twenty μL of 5M NaCl solution was added to the eluate and

the samples were incubated at 65°C for 4 hours to reverse cross-links. Following this incubation, 31.5µL of protease K inhibitor solution (Sigma-Aldrich) was added and incubated for 1 hour at 45°C to degrade proteins. Following this incubation, 500µL of phenol:chloroform (1:1) was added to each sample. The samples were vortexed vigorously for 30 seconds and centrifuged at 16,000 × g for 10 minutes at room temperature. The aqueous layer was carefully removed, transferred to a new tube, and 1.0mL of 100% ethanol and 150µL of 3M sodium acetate was added to each sample to induce DNA precipitation. The samples were mixed by inversion and were left in -20°C.

After 12 hours, DNA was precipitated by centrifuging at 16,000 × g for 10 minutes at 4°C. The supernatant was carefully removed. The pellet was washed once with 70% ethanol, re-centrifuged and dried. The pellet was resuspended in 8.0 pH EDTA for use in subsequent qPCR analysis.

Pulldown antibodies include anti-H3K27me3 and anti-Pol2 (Milipore). Control for these antibodies include IgG and ascitic fluid negative control. Primer design to the miR-200b promoter region was performed based on an identified promoter region 2kb upstream of the miR-200b gene (Sigma-Aldrich). Control primers provided by the manufacturer were used to assure the quality of the ChIP reaction, as well as to determine specificity of the chromatin modification changes associated with high glucose. Sequences for these primers are listed in Table 2.3.

Table 2.3 Oligonucleotides for ChIP-qPCR Analysis

Genomic region	Primer Sequences (5' → 3')
miR-200b promoter	GCCGGGATCACATTCCTC CTCAGCTTGGGAAAATCCAG
Human alpha satellite	CTGCACTACCTGAAGAGGAC GATGGTTCAACACTCTTACA
GAPDH promoter	TACTAGCGGTTTTACGGGCG TCGAACAGGAGGAGCAGAGAGCGA

2.6 PRC2 inhibition with 3-Deazaneplanocin A (DZNep) chemical inhibitor

A general methylation inhibitor DZNep (Cayman Chemical, Ann Arbor, MI), which has shown selectivity for PRC2 and H3K27me₃, was used as a loss-of-function treatment (80). DZNep is an indirect inhibitor of methylation, which functions by directly inhibiting a hydrolase that metabolizes Ado-Hcy. Ado-Hcy is an end product formed after a methyltransferase, like EZH2, uses its substrate, Ado-Met, to methylate an amino acid or nucleotide residue (50). DZNep therefore results in increased cellular levels of Ado-Hcy, which inhibits EZH2 by moving it to an inactive conformation. Concentrations from 1µM to 10µM have been shown to inhibit H3K27me₃ activity and 5µM was used for these experiments (90).

DZNep was initially dissolved in DMSO at a concentration of 10mM and was aliquoted for future use. DZNep was tested in normal glucose and high glucose, with an equal amount of DMSO (Santa Cruz Biotechnology, Santa Cruz, CA) as a control in the same conditions. Cells were pre-treated with DZNep at the starvation period, prior to addition of D-glucose, as well as during the D-glucose treatments.

2.7 PRC2 gene knockdown with small interfering RNA

To improve the specificity of the hypothesized mechanism, knockdown of specific gene targets was performed using small interfering (siRNA). Specifically, EZH2 and SUZ12 siRNA (Qiagen) were used for gene knockdown with control siRNA to control for the transfection process and specificity (Table 2.4).

Table 2.4 Silencing small interfering RNA sequences

Gene	siRNA sequences (5' → 3')
Control Primers	Life Technologies (AM4635)
EZH2	CCAUGUUUACAACUAUCAATT UUCAUACUUCUAAACAUGGTT
SUZ12	GCAUAAUGUCAAUAGAUAAAT UUAUCUAUUGACAUAUAUGCTA

Based on previous experiments in our laboratory, Lipofectamine was used in the following ratio based on past experiments in our laboratory (2.5 μ L Lipofectamine:1 μ g siRNA) with a final concentration of 100nM. To prepare this, siRNA and Lipofectamine 2000 (Invitrogen) was first mixed individually in Opti-MEM (Life Technologies) containing reduced serum and no antibiotics. After five minutes, the individual solutions were combined, mixed by inversion, allowed to settle for 30 minutes. Cells were grown to 80% confluency and were washed with D-PBS prior to transfection to remove antibiotics. One mL of Opti-MEM containing siRNA was added to cells. The cells were transfected for 4 hours and was supplemented with 2x the volume of full EBM-2 containing 10% FBS, growth factors and antibiotics. 24 hours later, the full medium was replaced with serum-free EBM-2 containing no growth factors or antibiotics and the cells were starved overnight. 18 hours later, cells were treated with D-glucose and were incubated for 48 hours, when they were collected for gene expression analysis as described above. Knockdown efficiency was measured by RT-PCR.

2.8 Tube Formation Assay

As a measure of VEGF activity and angiogenesis, a tube formation assay was performed with transfected cells. BD Matrigel Matrix Phenol Red-Free (BD Biosciences, Bedford, MA) was aliquoted (100 μ L per well) into a 96-well plate. The plate was spun briefly to remove bubbles and was incubated for 1 hour at 37°C. Prior to seeding, the transfected cells were collected with trypsin and cellular density was quantified using a hemocytometer. Once the Matrigel

solidified, cells were seeded at a density of 1.5×10^4 cells per well. Density of cells plated was determined empirically. The cells were then incubated for 1 hour at 37°C to allow for attachment, after which medium was aspirated and replaced with serum-free medium containing 25mM (HG) D-glucose. Cells transfected with control siRNA were also cultured in 5mM (NG) D-glucose as a control. The plate was then returned to 37°C to allow for tube formation. After 16 hours, the medium was carefully aspirated from each well, the cells were washed once with D-PBS and pictures were taken at $40\times$ magnification using a Nikon Diaphot microscope (Nikon Canada, Mississauga, ON) with PixelINK camera and PixelINK Capture OEM software (PixelINK, Ottawa, ON). Branch numbers and branch points were counted and a ratio of branches:branch points was calculated for each treatment. Each treatment was repeated in triplicate with at least two field of views per replicate.

2.9 Cell Viability Assay

To determine the cytotoxicity of the treatments used in our experiments, the WST-1 Cell Viability Assay (Roche) was used. Cells were seeded (5.0×10^4 /well) onto a 96-well plate and were allowed to attach overnight. The following morning, the full medium was removed and was replaced with 100 μL of serum-free medium containing various concentrations of D-glucose (5mM, 10mM, 15mM, 25mM, 50mM, 100mM) and various concentrations of DZNep (50nM, 500nM, 5 μM , 50 μM , 500 μM) with DMSO as a control (0.0005%, 0.005%, 0.05%, 0.5%, 5% v/v). Following 24 hours of incubation with the

agent, 10 μ L of WST-1 reagent was added to each well. The plate incubated for 1.5 hours at 37°C to produce a colour reaction. Absorbance was measured with a Multiskan FC Microplate Photometer (Thermo Scientific, Finland) at 450nm with a reference wavelength of 690nm. Survival was determined by the difference between the absorbances at 450nm and 690nm.

2.10 Statistical analysis

To determine statistical significance, 2-tailed Student's T-test was performed with an α -value of 0.05 using SPSS. P values < 0.05 were considered statistically significant (*). For experiments with multiple groups, one-way ANOVA was performed with an α -value of 0.05 using SPSS. Tukey's test was performed to determine differences between groups, with bars represented by different letters represented a significant difference, while a common letter denotes groups that are not significantly different.

Chapter 3: Results

3.1 High levels of glucose increase VEGF expression in HRMECs

Based on previous experiments performed in our laboratory with different endothelial cell types, specific concentrations and time points for glucose exposure were selected for *in vitro* analysis. However, since the human retinal microvascular endothelial cell (HRMEC) type is a relatively a new model in our laboratory, experiments were performed to confirm whether glucose increase VEGF levels. We show that VEGF mRNA levels was significantly increased after 24 hour exposure to medium containing 25mM glucose compared to 5mM glucose, while concentrations greater than 25mM did not show any differences in VEGF expression levels (Figure 3.1). The 25mM D-glucose exposure also showed decreased cell viability compared to 5mM D-glucose (Figure 3.2). Therefore, 5mM glucose was selected as normal glucose (NG) and 25mM glucose was selected as the high glucose (HG) for all subsequent analyses. These findings are consistent with previous studies performed in our laboratory using various endothelial cell types.

To test the effects of glucose at different time points, HRMECs were exposed to 5mM or 25mM D-glucose for various durations. VEGF transcript levels were significantly increased in HG compared to NG at 24 hour and 48 hours time points, though no significant changes were observed before these time points (Figure 3.3). Therefore, subsequent experiments were performed at these time points to test the effects of glucose exposure on endothelial cells.

Figure 3.1

Real time RT-PCR analysis of VEGF mRNA at different concentrations of glucose. HRMECs exposed to various concentrations of D-glucose for 24 hours exhibited differential mRNA levels of VEGF. Compared to 5mM D-glucose, VEGF expression was significantly increased at 15mM and 25mM D-glucose concentrations. VEGF was not increased at concentrations higher than 25mM. [* $p < 0.05$ compared to 5mM control; $n=4$; data expressed as mean \pm SEM, normalized to β -actin and expressed as a fold change of 5mM]

VEGF expression at various glucose concentrations

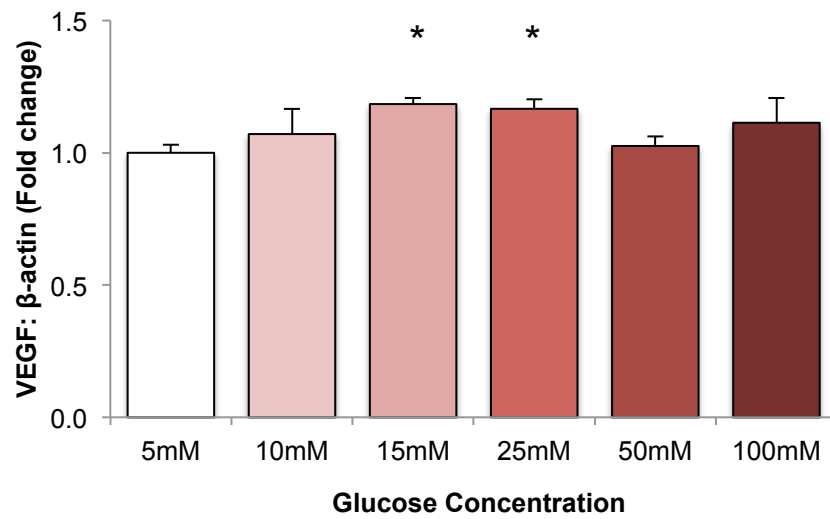


Figure 3.2

WST-1 cell survival assay at different glucose concentrations. HRMECs exposed to increasing concentrations of D-glucose for 24 hours exhibited decreased cell viability as measured by a WST-1 assay. Compared to 5mM D-glucose, cell viability was significantly decreased at 25mM, 50mM and 100mM. [* $p < 0.05$ compared to 5mM control; n=4; data expressed as mean \pm SEM]

HRMEC survival in different concentrations of glucose

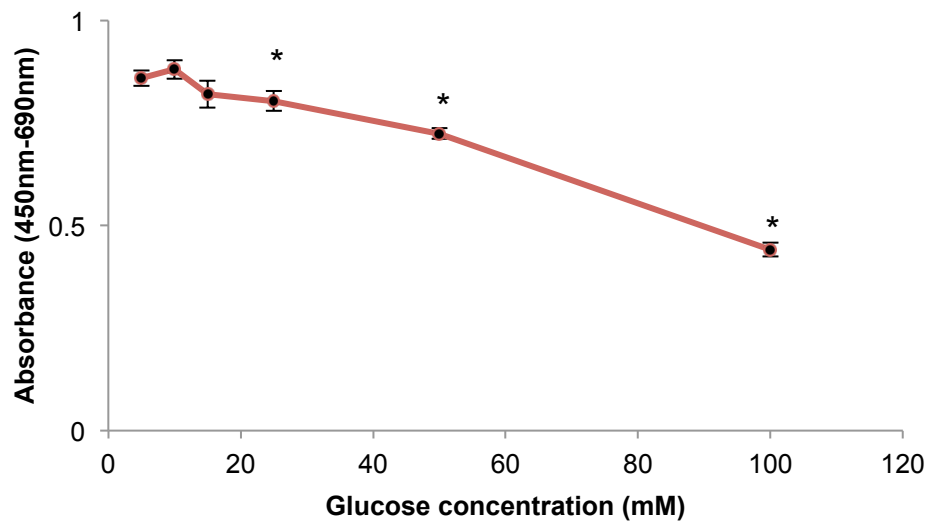
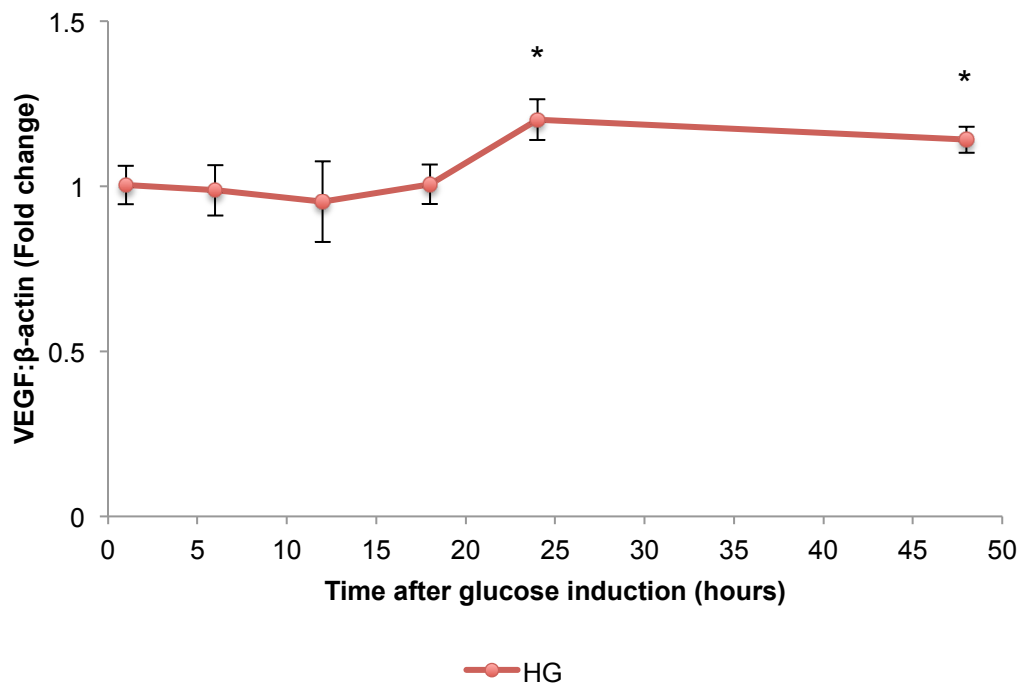


Figure 3.3

Real time RT-PCR analysis of VEGF mRNA levels at different durations of glucose exposure. HRMECs exposed to 25mM (high glucose; HG) glucose for 24 and 48 hours demonstrated significantly increased VEGF mRNA compared to 5mM (normal glucose; NG). These differences were not observed at time points earlier than 24 hours. [* $p < 0.05$ compared to NG; $n=4$; data expressed as mean \pm SEM, normalized to β -actin and expressed as a fold change of NG within each time point]

VEGF expression between NG and HG at various time points



3.2 miR-200b and VEGF are altered in human retinal endothelial cells exposed to high levels of glucose

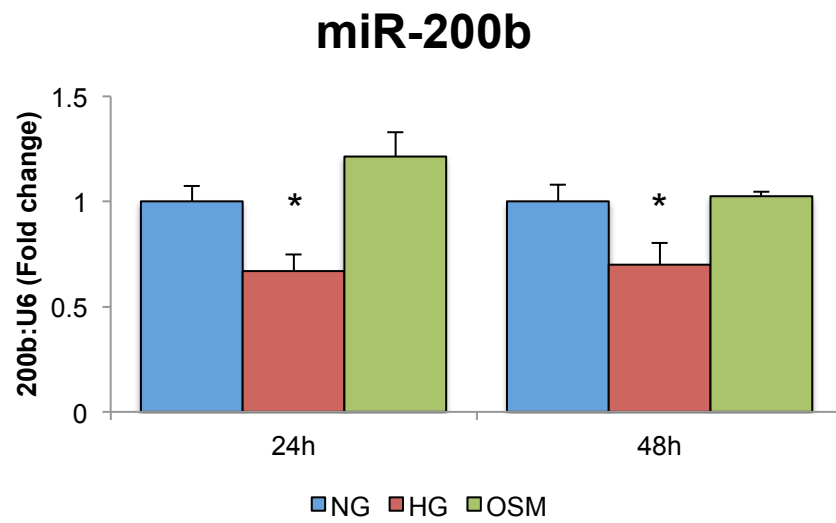
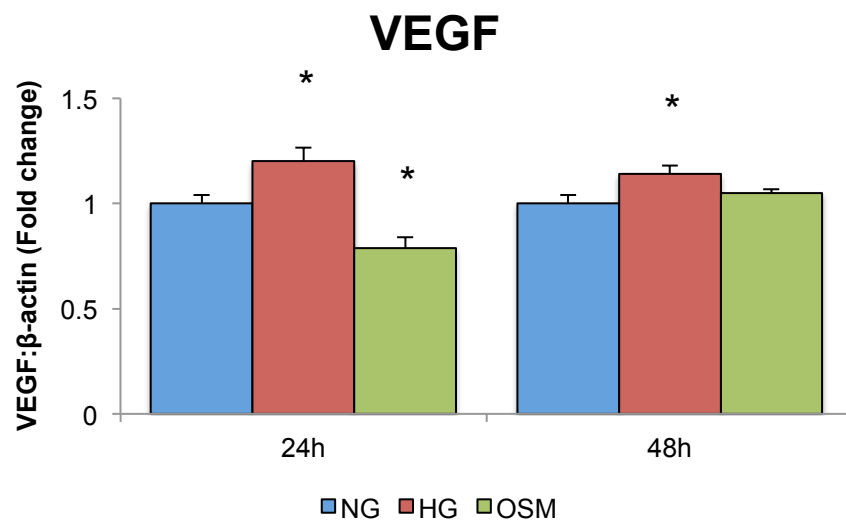
To verify the effects of glucose exposure on miR-200b expression, HRMECs were exposed to 25mM D-glucose for 24 and 48 hours. An equimolar L-glucose treatment (osmotic control; OSM) was also used. L-glucose is metabolically inactive and does not cause oxidative stress and hyperglycemia-induced signaling changes as D-glucose does (91,92). At both 24 and 48 hours, miR-200b levels showed significant decrease in HG compared to NG and OSM controls (Figure 3.4A). In parallel, VEGF showed significantly increased levels in HG compared to NG and OSM controls (Figure 3.4B). This result is consistent with previous work performed in our laboratory in bovine retinal endothelial cells. This is the first time miR-200b decrease has been observed in human endothelial cells isolated from the retina. This finding further strengthens the relationship between hyperglycemia and decreased miR-200b expression in the context of diabetic retinopathy.

3.3 PRC2 components are altered in endothelial cells exposed to high glucose levels

Once decreased miR-200b and increased VEGF was established in HRMECs exposed to HG, the mRNA levels of several genes associated with the PRC2 complex was measured by qPCR. These included EZH2, the methyltransferase portion of PRC2, EED and SUZ12, the adaptor protein of

Figure 3.4

Real time RT-PCR analysis of miR-200b (A) and VEGF (B) in HRMECs exposed to 5mM D-glucose (NG) 25mM D-glucose (HG) and 20mM L-glucose+5mM D-glucose (osmotic control; OSM). HRMECs cultured for 24 hours and 48 hours in HG showed significantly decreased levels of miR-200b with parallel increased levels of VEGF expression compared to NG and OSM. [* $p < 0.05$ compared to NG; n=6; data expressed as mean \pm SEM, normalized to β -actin and expressed as a fold change of NG].

A**B**

PRC2, and KDM6A and KDM6B, which are the demethylases associated with H3K27me3 and thus oppose PRC2 activity.

At both 24 and 48 hours in HG, mRNA of EZH2, EED and SUZ12 were elevated compared to NG and OSM controls (Figure 3.5). These observations show increased expression of the PRC2 components, which were hypothesized to negatively regulate miR-200b expression. Thus, this finding supports the hypothesis by correlating the increased expression of the PRC2 components with the decrease in miR-200b.

Furthermore, no difference was observed in expression levels of KDM6A and KDM6B between NG, HG and OSM controls (Figure 3.6). Interestingly, KDM6A was observed to significantly decrease in HG at the 48 hour time point (Figure 3.6A). This suggests there is no change or even a decrease in demethylase expression for H3K27 methylation. Altogether, an increase in expression of PRC2 with no change or decrease in demethylase expression suggests a shift towards increase of H3K27me3 in HG. Overall, these experiments provide correlational evidence linking PRC2 to negatively regulating miR-200b in HG, though additional supporting evidence is necessary.

3.4 PRC2 activity is increased specifically at the miR-200b promoter region

To demonstrate the involvement of PRC2 at the level of the genome and chromatin modifications, ChIP was performed using antibodies for H3K27me3. Negative control antibody IgG was used to demonstrate specificity of the immunoprecipitation reaction. RNA Polymerase 2 (Pol2) was also

Figure 3.5

Real time RT-PCR analysis of PRC2 components in HRMECs exposed to 5mM D-glucose (NG), 25mM D-glucose (HG), and 20mM L-glucose+5mM D-glucose (OSM). After 24 and 48 hour exposure to D-glucose, levels of EZH2, EED and SUZ12, were significantly increased compared to NG and OSM controls. [* $p < 0.05$ compared to NG; $n=6$; data expressed as mean \pm SEM, normalized to β -actin and expressed as a fold change of NG].

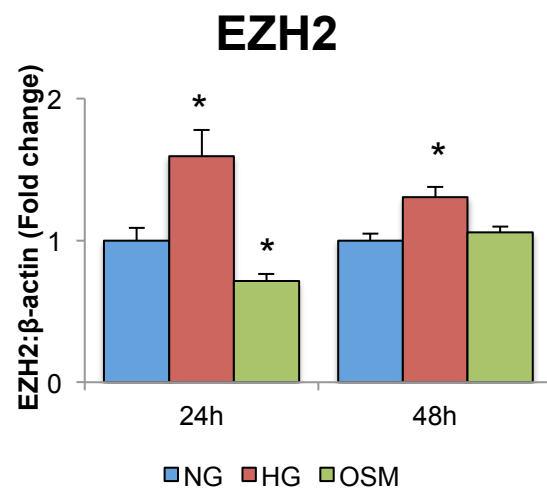
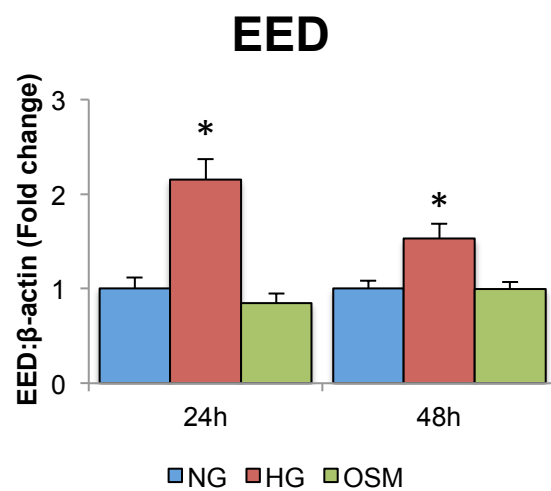
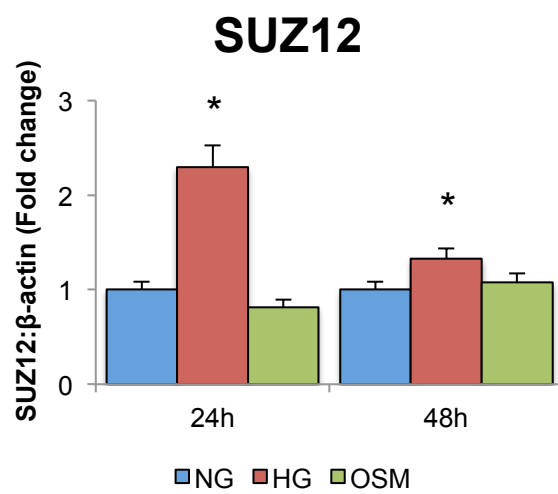
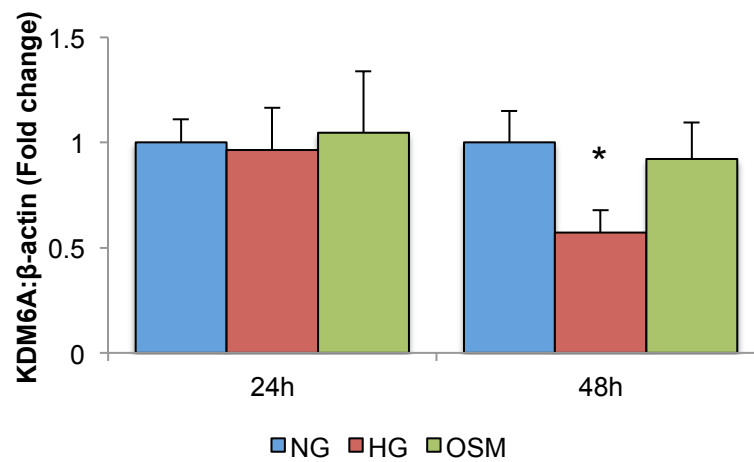
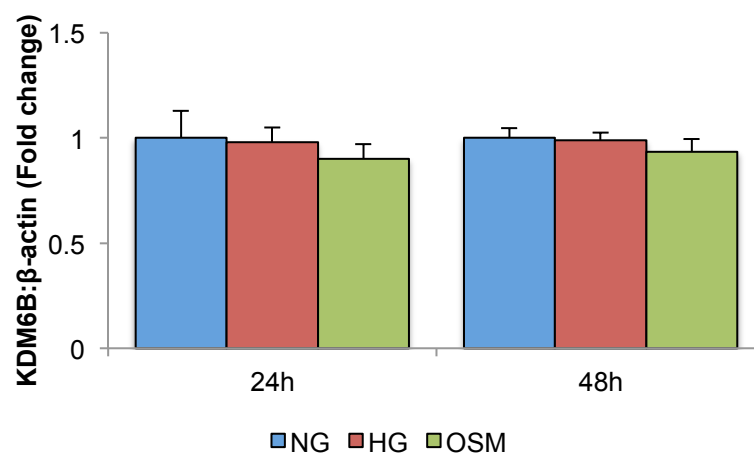
A**B****C**

Figure 3.6

Real time RT-PCR analysis of demethylases for H3K27me3 in HRMECs exposed to 5mM D-glucose (NG), 25mM D-glucose (HG), and 20mM L-glucose+5mM D-glucose (OSM). After 24 exposure to D-glucose, KDM6A and KDM6B levels did not change in HG. After 48 hours, KDM6A was significantly decreased compared to NG and OSM controls, while KDM6B showed no change. [* $p < 0.05$ compared to NG; $n=6$; data expressed as mean \pm SEM, normalized to β -actin and expressed as a fold change of NG].

A**KDM6A****B****KDM6B**

immunoprecipitated to measure transcriptional activity of miR-200b at the level of the genome in addition to the RT-PCR results demonstrated above. Ascitic was used as a negative control for this antibody as provided by the manufacturer.

Following immunoprecipitation and qPCR analysis, H3K27me3 was found to be significantly increased at the miR-200b promoter region in HG compared to NG controls (Figure 3.7). IgG isotype control pull down showed significantly less association than the H3K27me3-specific antibody. Also, Pol2 was found to be significantly decreased at the miR-200b promoter region in HG compared to NG controls (Figure 3.8). Again, specificity of this antibody was demonstrated as association with the negative control was minimal. Therefore, this increase of the H3K27me3, which is specific to the PRC2 complex, demonstrates a strong regulatory relationship between PRC2 and miR-200b at the level of chromatin modification. Furthermore, since this type of methylation is believed to be repressing and is associated with closed chromatin, the observation of decreased Pol2 association at the miR-200b promoter region further supports the mechanism and accounts for the decrease in miR-200b expression demonstrated earlier in this project. Altogether, these results paint a picture of the genomic events occurring at the miR-200b promoter region in HG, and further support that PRC2 may regulate miR-200b in diabetes.

Finally, to improve the specificity of this mechanism, areas of the genome with well-known association to the targets that were immunoprecipitated were measured by qPCR. No significant differences were observed in H3K27me3 and Pol2 association at the α -satellite region and GAPDH promoter region between

Figure 3.7

ChIP-qPCR analysis of H3K27 trimethylation (H3K27me3) at the miR-200b promoter region. Association of H3K27me3 at the miR-200b promoter region was significantly increased in 25mM (HG) glucose compared to 5mM (NG) glucose. Association of IgG negative control antibody was minimal and showed no difference between NG and HG. [* $p < 0.05$ compared to NG; $n=3$; data expressed as mean percentage of input \pm SEM].

Relative H3K27 trimethylation at miR-200b promoter region

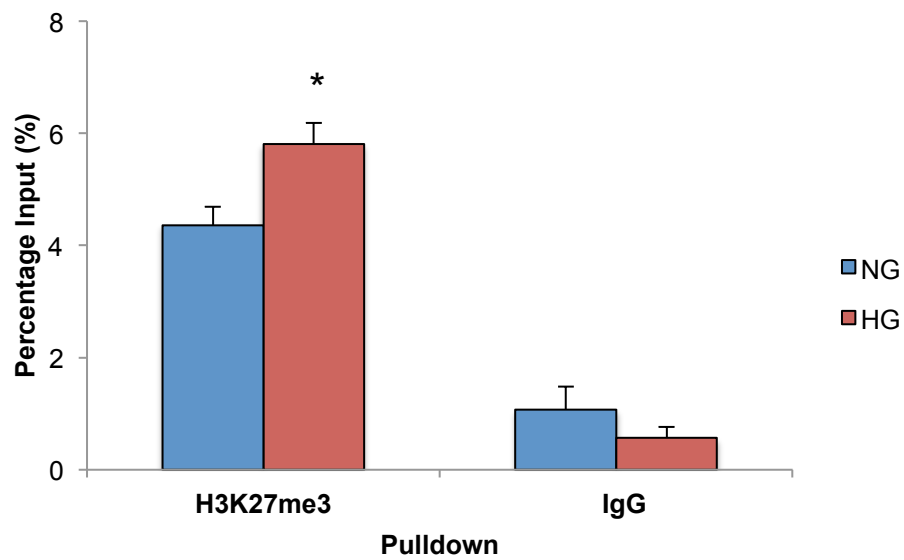
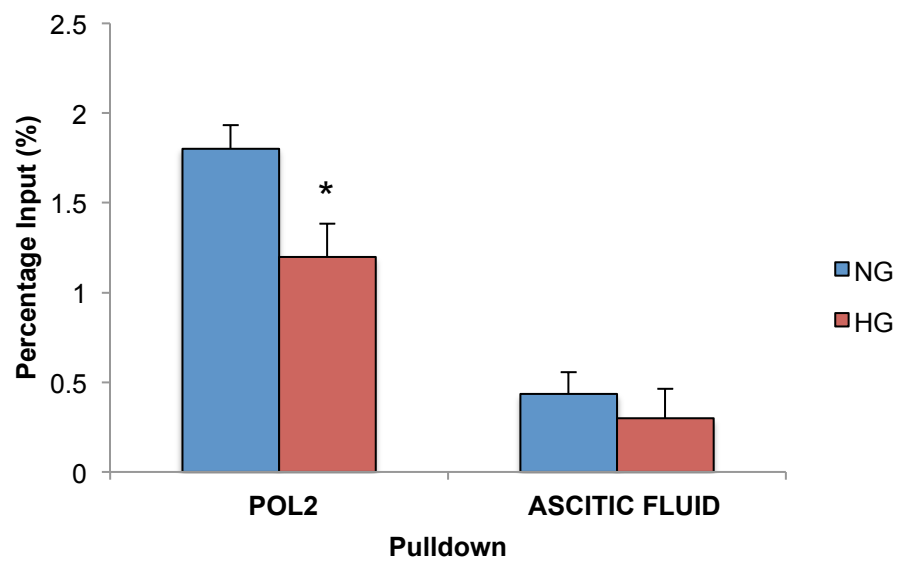


Figure 3.8

ChIP-qPCR analysis of RNA polymerase 2 (Pol2) at the miR-200b promoter region. Association of Pol2 at the miR-200b promoter region was significantly decreased in 25mM (HG) glucose compared to 5mM (NG) glucose. Association of ascitic fluid (negative control) was minimal and showed no difference between NG and HG. [* $p < 0.05$ compared to NG; $n=3$; data expressed as mean percentage of input \pm SEM].⁵

Relative Pol2 association at miR-200b promoter region



NG and HG, though specificity between the specific and non-specific antibodies was still observed (Figures 3.9 and 3.10). These negative control experiments further support that PRC2 specifically regulates miR-200b in HG, as these changes in H3K27me3 and Pol2 association are specific to certain genomic regions and are not global.

3.5 Loss of function analysis using DZNep inhibitor demonstrates a cause-and-effect relationship between PRC2 and miR-200b expression

To demonstrate a cause-and-effect relationship between PRC2 and miR-200b, a chemical inhibitor for H3K27me3, DZNep, was used. Prior to experimenting with DZNep, the potential toxicity of different concentrations of DZNep was measured using a WST-1 cell viability assay. Compared to DMSO control, DZNep showed increased survival at all concentrations (Figure 3.11). Significantly increased toxicity was observed at 500 μ M DZNep and 5% DMSO. The concentration of 5 μ M was selected for further studies.

HRMECs treated for 24 hours with DZNep showed significantly higher miR-200b levels in both NG and HG compared to the other controls (Figure 3.12A). In parallel, VEGF mRNA was significantly decreased when HRMECs were treated with DZNep compared to all other controls (Figure 3.12B). Since PRC2 was hypothesized to negatively inhibit miR-200b, through its repressing methylation-mark, increased expression of miR-200b would be expected by inhibiting PRC2. Increased miR-200b expression was observed with decreased

Figure 3.9

ChIP-qPCR analysis of H3K27 trimethylation (H3K27me3) at the human alpha-satellite region. Association of Pol2 at the human alpha satellite region showed no significant change between 25mM (HG) glucose and 5mM (NG) glucose.

Association of IgG negative control antibody was minimal and showed no difference between NG and HG. [* $p < 0.05$ compared to NG; $n=3$; data expressed as mean percentage of input \pm SEM].

Relative H3K27 trimethylation at alpha-satellite region

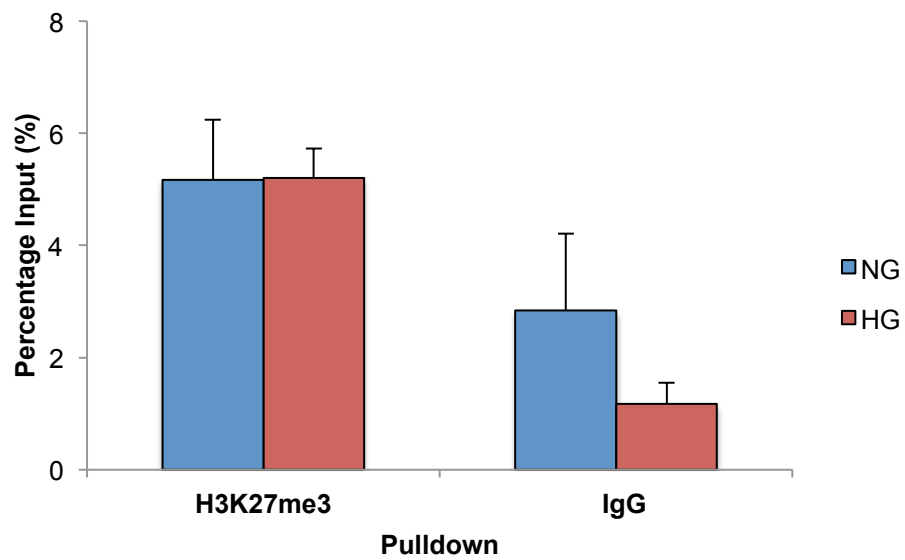


Figure 3.10

ChIP-qPCR analysis of RNA polymerase 2 (Pol2) at the GAPDH promoter region. Association of Pol2 at the GAPDH promoter region showed no significant difference between 25mM (HG) glucose and 5mM (NG) glucose. Association of ascitic fluid (negative control) was minimal and showed no difference between NG and HG. [* $p < 0.05$ compared to NG; $n=3$; data expressed as mean percentage of input \pm SEM].

Relative Pol2 association at GAPDH promoter region

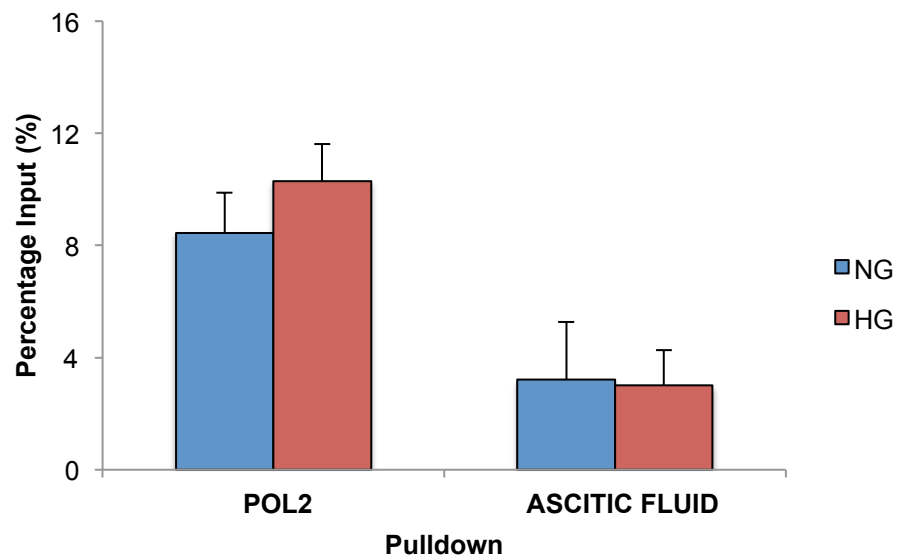


Figure 3.11

WST-1 cell viability assay shows that HRMECs exposed to 3-Deazaneplanocin A (DZNep) for 24 hours increased in viability compared to an equivalent concentration of DMSO. [* $p < 0.05$ compared to DMSO control; $n=4$; data expressed as mean \pm SEM].

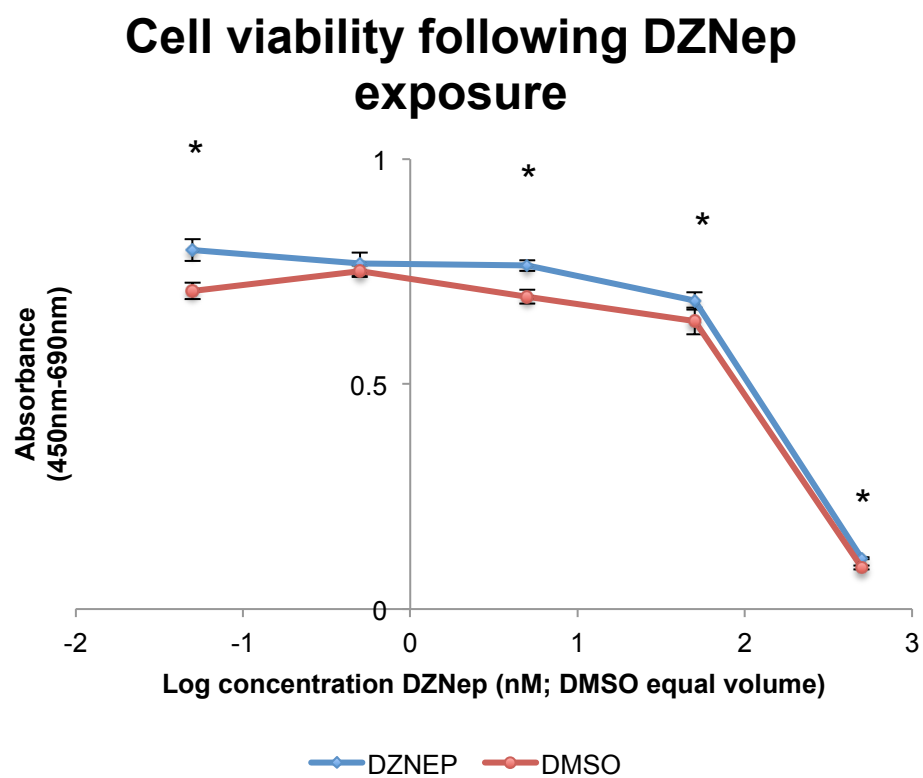
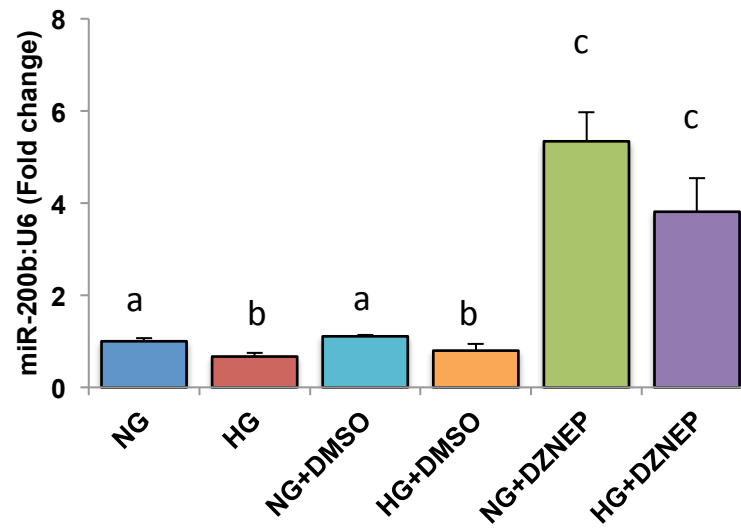
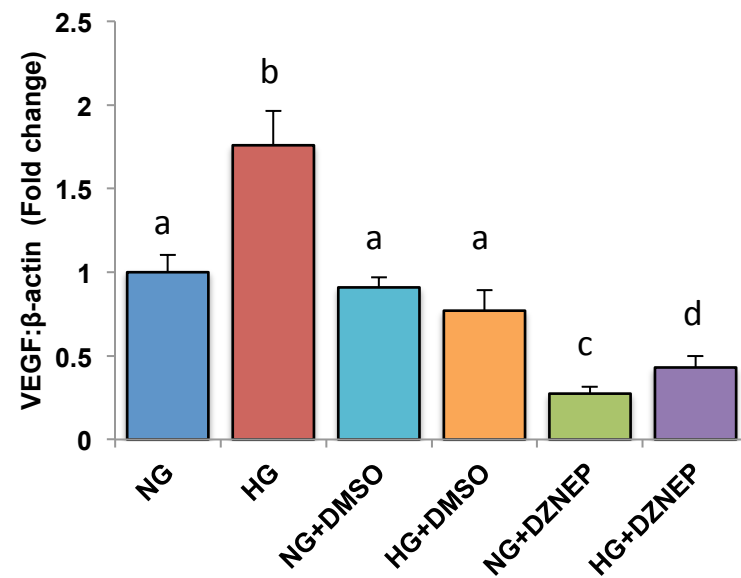


Figure 3.12

Real time RT-PCR analysis of miR-200b and VEGF levels in HRMECs treated with 3-Deazaneplanocin A (DZNep) in 25mM (HG) glucose or 5mM (NG) glucose). After 24 exposure to DZNep in NG and HG, miR-200b levels were significantly increased with parallel decreased levels of VEGF compared to controls. [NG = 5mM D-glucose, HG = 25mM D-glucose, NG+DMSO = 5mM D-glucose + 0.05% DMSO, HG+DMSO= 25mM D-glucose + 0.05% DMSO, NG+DZNEP = 5mM D-glucose + 5 μ M DZNep, HG+DZNEP = 25mM + 5 μ M DZNep; identical letters represent groups that are not significantly different; $p < 0.05$; $n=6$; data expressed as mean \pm SEM, normalized to β -actin for VEGF and U6 for miR-200b, expressed as a fold change of NG].

A**miR-200b with PRC2 inhibitor****B****VEGF with PRC2 inhibitor**

VEGF mRNA, a target of miR-200b, providing evidence to support the hypothesis by showing a cause-and-effect relationship by using loss-of-inhibition.

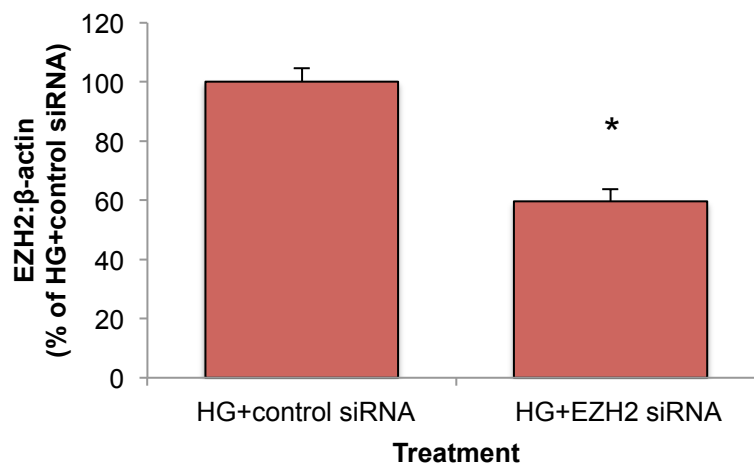
3.6 Loss of function with siRNA demonstrates that SUZ12 is of importance in PRC2-mediated regulation of miR-200b

To further elucidate the role of specific components of PRC2 in the regulation of miR-200b, siRNA-mediated gene knockdown of EZH2 and SUZ12 was performed. Control siRNA was used as a negative control. Knockdown efficiency was verified by RT-PCR and efficacy was verified by measuring the transcript levels of a known target of PRC2 (Figures 3.13 and 3.14). In HG, HRMECs transfected with control siRNA showed a significant decrease in miR-200b expression and increase in VEGF expression (Figure 3.15). When EZH2 was silenced in HG, miR-200b and VEGF showed no differences in expression compared to HRMECs treated with control siRNA in HG (Figure 3.15). However, silencing of SUZ12 increased miR-200b and decreased VEGF, with levels similar to HRMECs transfected with control siRNA in NG (Figure 3.15). In addition, a tube formation assay was conducted to provide a functional correlate. HRMECs transfected with control siRNA and EZH2 siRNA in HG showed significantly increased branching compared to HRMECs transfected with control siRNA in NG (Figure 3.16). HRMECs treated with SUZ12 siRNA in HG showed decreased branching, equivalent to HRMECs treated with control siRNA in NG (Figure 3.16). Altogether, this data suggests that SUZ12 is important in regulating miR-200b in HG, as knockdown of SUZ12 corrected miR-200b and VEGF levels.

Figure 3.13

Real time RT-PCR analysis of EZH2 and SUZ12 in HRMECs transfected with control or specific siRNA. When cultured in 25mM (HG) glucose, HRMECs transfected with EZH2 siRNA showed significantly decreased levels of EZH2 compared to HRMECs transfected with control siRNA. Furthermore, HRMECs transfected with SUZ12 siRNA showed significantly decreased levels of SUZ12 compared to HRMECs transfected with control siRNA. [* $p < 0.05$ compared to HG+control siRNA; n=6; data expressed as mean \pm SEM, normalized to β -actin and expressed as a fold change of HG+control siRNA].

A EZH2 Knockdown Efficiency



B SUZ12 Knockdown Efficiency

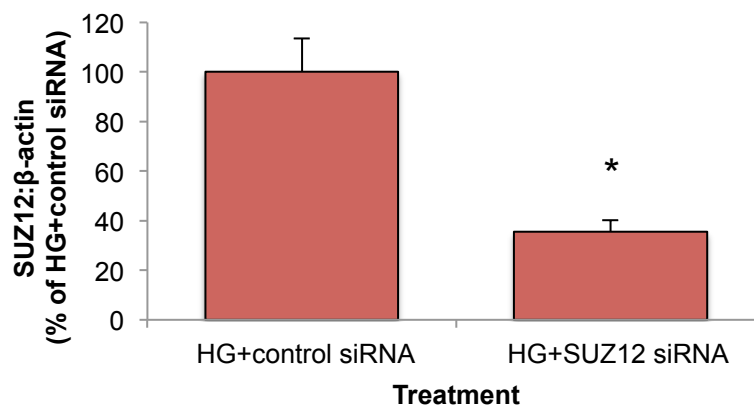


Figure 3.14

Real time RT-PCR analysis of VASH1 in HRMECs exposed to 25mM (HG) glucose with EZH2 or SUZ12 siRNA. VASH1 mRNA levels were significantly increased in HRMECs transfected with EZH2 and SUZ12 siRNA compared to HRMECs transfected with control siRNA. [* $p < 0.05$ compared to HG+control siRNA; n=6; data expressed as mean \pm SEM, normalized to β -actin and expressed as a fold change of HG+control siRNA].

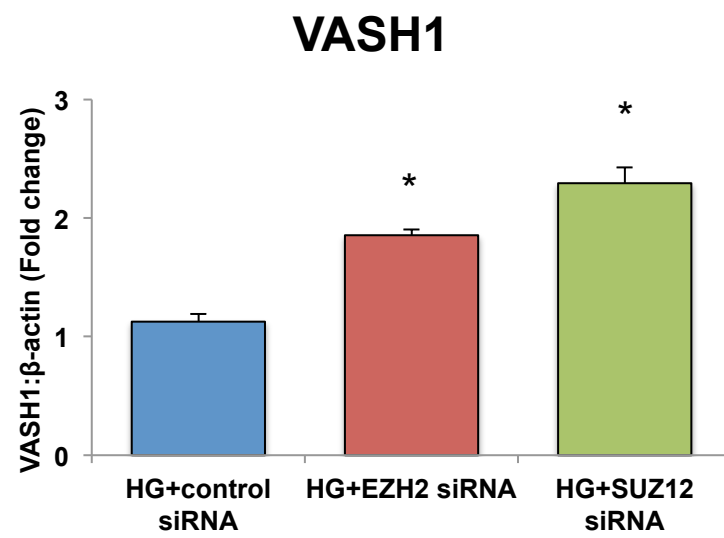


Figure 3.15

Real time RT-PCR analysis of miR-200b and VEGF levels in HRMECs transfected with EZH2 or SUZ12 siRNAs after 48 hour exposure to 25mM (HG) glucose or 5mM (NG) glucose). In HRMECs transfected with EZH2 siRNA in HG, miR-200b and VEGF expression was not significantly different from HG+control siRNA but decreased compared to NG+control siRNA. In HRMECs transfected with SUZ12 siRNA in HG, miR-200b was significantly increased with parallel decreased levels of VEGF compared to HRMECs transfected with control siRNA, with levels similar to NG+control siRNA. [NG+control siRNA = 5mM D-glucose + 100nM control siRNA, HG+control siRNA = 25mM D-glucose + 100nM control siRNA, HG+EZH2 siRNA = 25mM D-glucose + 100nM EZH2 siRNA, HG+SUZ12 siRNA = 25mM + 100nM SUZ12 siRNA; identical letters represent groups that are not significantly different; $p < 0.05$; $n=6$; data expressed as mean \pm SEM, normalized to U6 or β -actin and expressed as a fold change of NG+control siRNA].

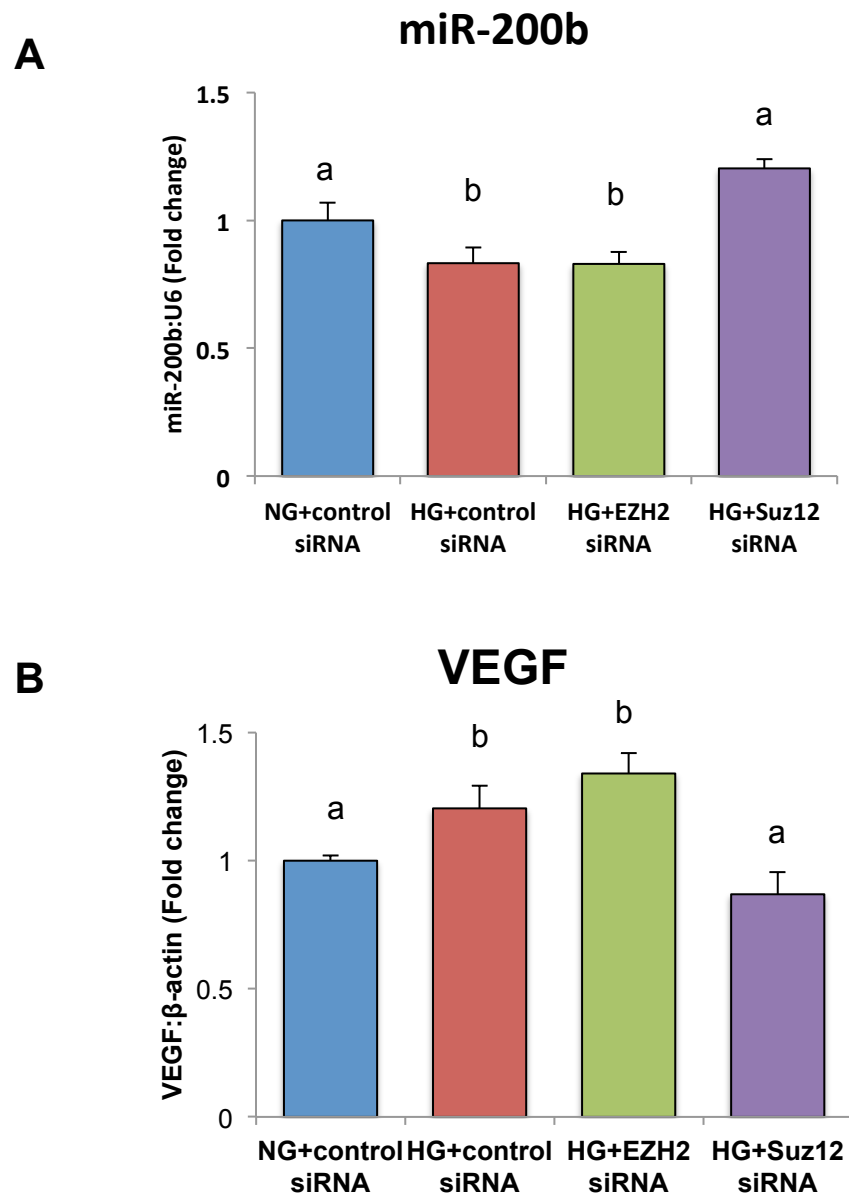
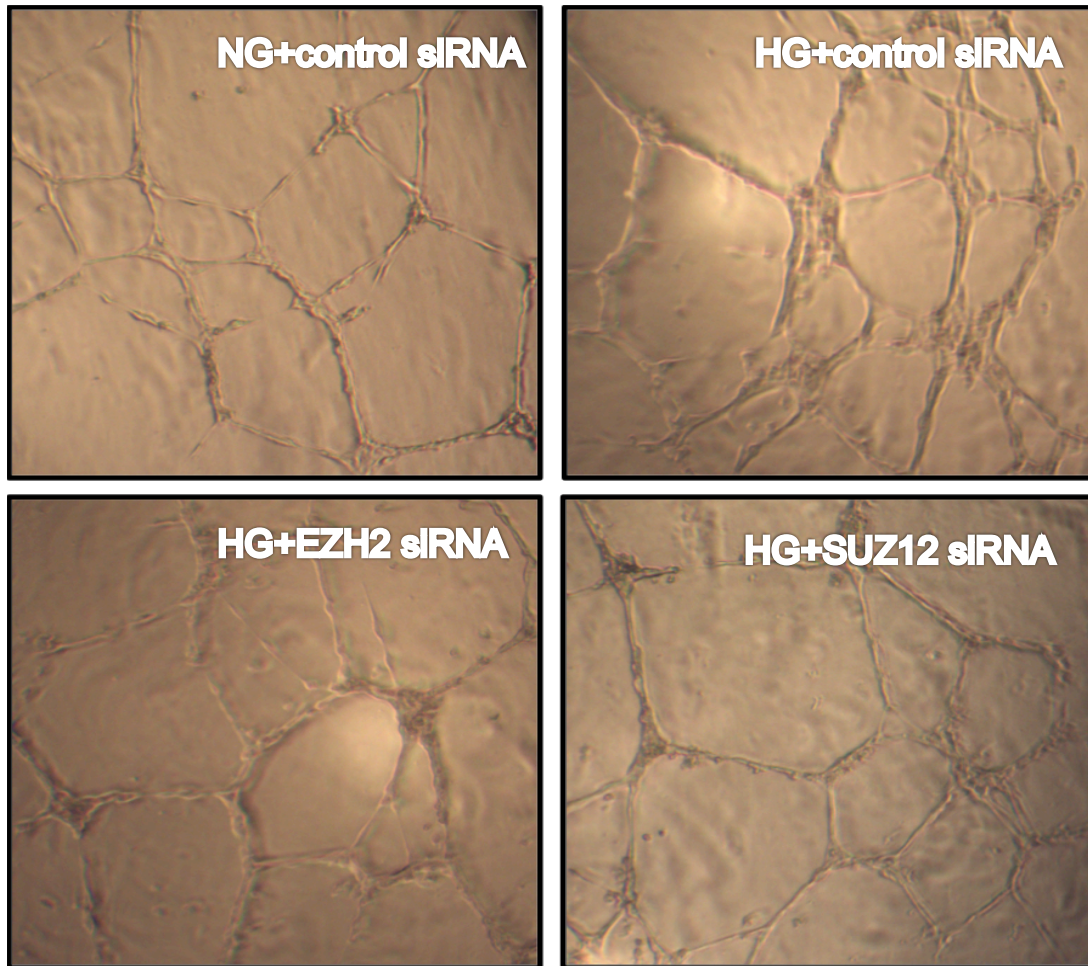
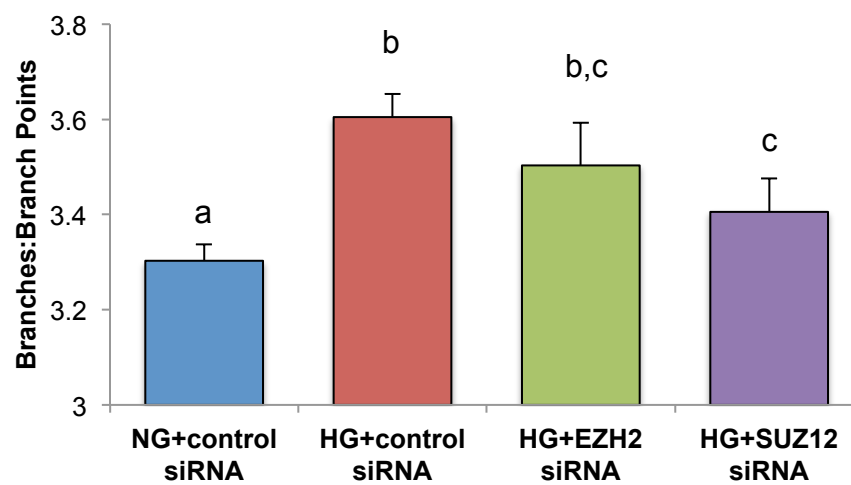


Figure 3.16

Tube formation assay as a measure endothelial branching in HRMECs transfected with various siRNA. 1.5×10^4 were seeded on top of Matrigel and incubated for 16 hours at 37°C to assess tubule formation. HRMECs transfected with HG+control siRNA demonstrated significantly increased branching compared to NG+control siRNA. Transfection of EZH2 siRNA did not reduce endothelial branching significantly compared to HG+control siRNA. However, transfection of SUZ12 siRNA significantly reduced endothelial branching compared to HG+control siRNA. [NG+control siRNA = transfected with 100nM control siRNA and incubated for 16h in 5mM D-glucose following seeding, HG+control siRNA = transfected with 100nM control siRNA and incubated for 16h in 25mM D-glucose following seeding, HG+EZH2 siRNA = transfected with 100nM EZH2 siRNA and incubated for 16h in 25mM D-glucose following seeding, HG+SUZ12 siRNA = transfected with 100nM SUZ12 siRNA and incubated for 16h in 25mM D-glucose following seeding; identical letters represent groups that are not significantly different; $p < 0.05$; $n=3$ biological replicates with 2 fields of view each; data expressed as mean \pm SEM].

A**B**

Tube Formation Assay Quantification



3.7 PRC2 involvement in other endothelial cell types and in target tissues of diabetic complications

Finally, the expression of PRC2 components was measured in retinal tissues of animal models of diabetes. We used streptozotocin-induced (STZ) rat and mouse models. All clinical data on these models is previous published (93,94). We used RNA isolated from retinal tissues of these models to determine whether changes seen in retinal endothelial cells are also present *in vivo*. EZH2, EED and SUZ12 were significantly increased in the retinal tissue of diabetic animals compared to non-diabetic controls (Figure 3.17). This finding is consistent with the *in vitro* data produced in this project and suggests that PRC2-mediated regulation of miR-200b may be relevant *in vivo*.

Furthermore, the expression of PRC2 components was measured in human dermal microvascular endothelial cells isolated from healthy individuals (HDMECs), from a patient with type 1 diabetes (Type 1) and a patient with type 2 diabetes, all treated in NG or HG. Significantly increased expression of PRC2 was observed in all cell types treated in HG (Figure 3.18). This suggests that PRC2 may be relevant in endothelial cells and may not be specific to retinal endothelial cells. This opens up the exciting possibility of common pathogenic mechanisms in diabetic complications.

Figure 3.17

Real time RT-PCR analysis of PRC2 component expression in animal retinal tissue from streptozotocin (STZ) induced diabetic and control animals. After 1 month of diabetes, EZH2, EED and SUZ12 levels were increased in rat retinal tissue from diabetic animals compared to control animals. After 2 months of diabetes, EZH2, EED and SUZ12 levels were increased in mouse retinal tissue of diabetic animals compared to controls. [* $p < 0.05$ compared to control; $n=6$; data expressed as mean \pm SEM, normalized to β -actin and expressed as a fold change of control].

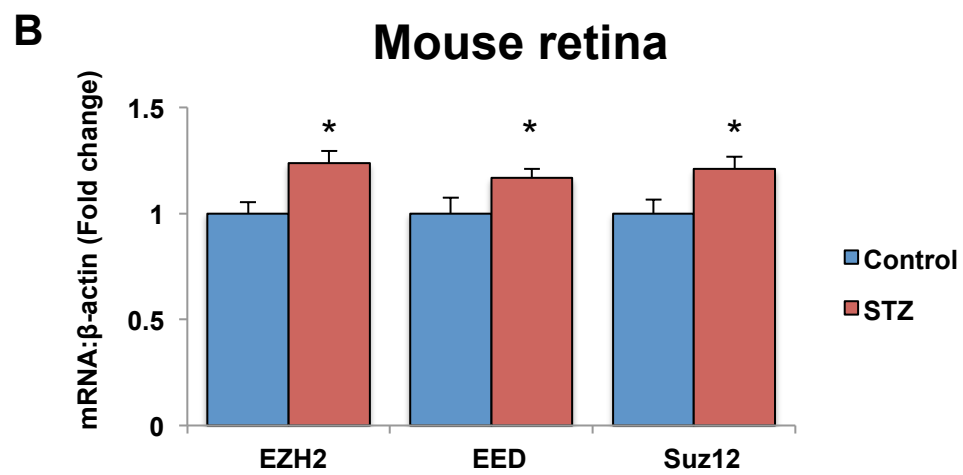
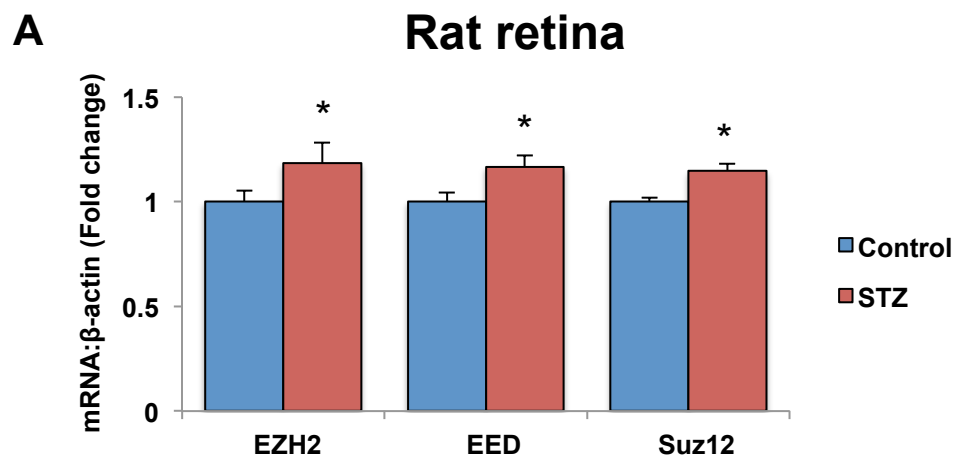
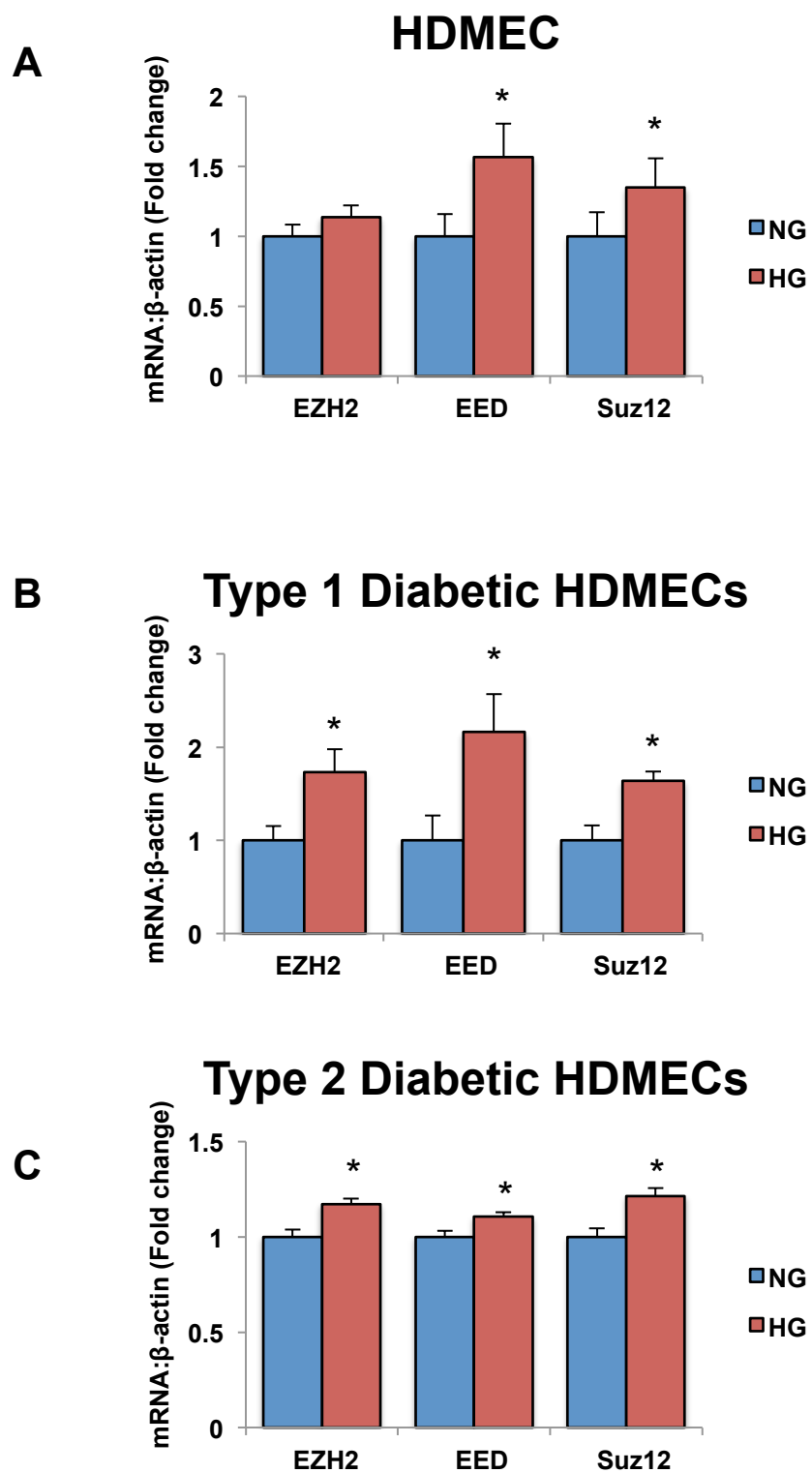


Figure 3.18

Real time RT-PCR analysis of PRC2 components in human dermal microvascular endothelial cells (HDMECs) of various origins. In non-diabetic adult HDMECs, EED and SUZ12 levels were significantly increased by HG. In HDMECs isolated from patients with diabetes (Type 1 & Type 2), EZH2, EED and SUZ12 were significantly increased in HG compared to NG controls.

[HDMECs = human dermal microvascular endothelial cells, Type 1 = HDMECs isolated from a patient with type 1 diabetes, Type 2 = HDMECs isolated from a patient with type 2 diabetes; * $p < 0.05$ compared to NG; $n=6$; data expressed as mean \pm SEM, normalized to β -actin and expressed as a fold change of NG].



Chapter 4: Conclusions

Discussion

Our key findings are: 1) miR-200b and VEGF are altered in HRMECs exposed to glucose, 2) PRC2 component mRNA and activity at the miR-200b promoter region is in HRMECs exposed to glucose, 3) treatment with DZNep inhibitor and transfection of SUZ12 siRNA increased miR-200b and decreased VEGF levels, and 4) PRC2 components are altered in retinal tissues from diabetic animals and other endothelial cell types exposed to high glucose. Histone methylation as a mechanism for a dynamic response to external stimuli, like hyperglycemia, is a new way of thinking. Few mediators and types of methylation have been studied in the context of diabetic complications thus far. H3K27 methylation research has been expanding in the cancer field but remains uninvestigated in diabetes. Furthermore, recent evidence from cancer research suggests that the major methyltransferase complex for H3K27 trimethylation, PRC2, is involved in silencing several miRNA involved in cancer, including miR-200b. Since miR-200b is also involved in diabetic retinopathy, a similar underlying mechanism may be present and is worth investigating.

HRMECs exposed to high glucose levels typically seen in diabetes showed decreased miR-200b with parallel increased VEGF. As mentioned earlier, there has been some controversy over the directionality of whether miR-200b becomes repressed or overexpressed in diabetic retinopathy. Different investigations have found conflicting results, although differences in animal model choice and cell culture conditions may account for this (95). Never the less, replicating miR-200b repression in high glucose conditions was an

important starting point in this study and verified results produced previously in our laboratory. Furthermore, this investigation showed repressed miR-200b in a relatively new cell type, which was isolated from human retina. This further strengthens the relevance of this *in vitro* model to the *in vivo* disease, as organ-specific signaling is sometimes apparent in diabetes (ie. not all organs respond in the same manner to hyperglycemia). Therefore, this cell type was chosen for all subsequent experiments for its relevance to the organ of interest.

Establishment of correlational evidence between miR-200b repression and PRC2 was important to first investigating a potential regulatory relationship. Though many proteins and lncRNA are involved in the PRC2 complex, three major components were selected for investigation. EZH2 is the major methyltransferase component while EED and SUZ12 are the major adaptor proteins. Expression of the major PRC2 components was increased in HG. Furthermore, this increase was specific to D-glucose treatment but not L-glucose. This suggests that PRC2 expression is increased due to signaling induced by hyperglycemia, and not simply due to osmolarity changes. This could be due to induction of certain transcription factors. For example, hypoxia inducible factor 1 alpha (HIF-1 α) has been shown to become stabilized and increase in transcriptional activity by high glucose in brain endothelial cells (96). Furthermore, HIF-1 α has been shown to regulate EZH2 and may be responsible for regulating others genes in the PRC2 complex (97,98). Further investigation into HIF-1alpha stabilization would be suggest as a possible upstream mechanism of PRC2 induction in hyperglycemia.

Interestingly, after 24 hours of glucose exposure, no change was observed in the demethylases for H3K27me3, KDM6A and KDM6B. Furthermore, after 48 hours of glucose treatment, KDM6A was decreased relative to NG controls, while KDM6B again showed no change. Although increased mRNA expression of PRC2 components, and no change or decrease in KDM6A/B mRNA, does not imply a direct increase in their activity, it does suggest that PRC2 activity may be increased through increased expression. Further investigating these components at the protein level would strengthen this relationship between high glucose and increased PRC2 expression.

Most importantly, H3K27me3 was increased in HG specifically at the miR-200b promoter region, just upstream of the transcriptional start site. This increase in H3K27 methylation, a repressing type of methylation, suggests formation of closed, inaccessible chromatin and accounts for the decreased expression of miR-200b. Since PRC2 mediates this type of methylation, this observation further strengthens the hypothesis that PRC2 regulates miR-200b in response to hyperglycemia. Furthermore, RNA Pol2 association at the same promoter region was decreased in HG, further suggesting that heterochromatin was formed and that decreased expression of miR-200b in HG is regulated at the genome level. Finally, these changes in qPCR appear to be specific to the miR-200b promoter region. At the human alpha-satellite region, an area of heterochromatin well known to associate with H3K27me3, no changes were observed in H3K27me3. Also, at the GAPDH promoter region, a highly expressed area of the genome, no changes were observed in Pol2 association. Therefore, H3K27me3 is increased

specifically, while Pol2 association is decreased specifically, at the miR-200b promoter region. This is one of the first investigations to connect two epigenetic mechanism, ie. histone methylation and microRNAs, in the context of a complication of diabetes. While there is no doubt that PRC2 regulates other genes, miR-200b is one gene that becomes repressed in response to PRC2 induction in HG.

ChIP-qPCR is a powerful technique for elucidating changes in chromatin modifications and transcriptional machinery recruitment. However, ChIP-qPCR is limited in the range of which changes can be determined because primers must be designed to specific regions. By combining ChIP with DNA sequencing (ChIP-seq), a greater overall picture of the genomic landscape can be mapped, providing information on how the overall miR-200 gene is affected, as well as if H3K27me3 affects other genes.

Cause-and-effect experimentation was important to further demonstrating a regulatory relationship between PRC2 and miR-200b. Using a chemical inhibitor of PRC2 and H3K27me3, DZNEP, miR-200b expression was significantly increased while VEGF was decreased in parallel. DZNep is not specific for H3K27me3 inhibition, thus siRNA-mediated gene knockdown was used to further elucidate the mechanism (90). siRNA against EZH2 and SUZ12 were used because these targets have been directly linked to regulating miR-200b in neoplasia. Interestingly, silencing of EZH2 in HG showed no significant changes in miR-200b and VEGF levels when compared to control siRNA. However, in HG, silencing of SUZ12 produced significantly increased miR-200b

and decreased VEGF expression compared to control siRNA, and similar expression to control siRNA in NG. Furthermore, silencing of SUZ12 produced decreased endothelial branching compared to control siRNA and EZH2 siRNA, indicating functional alterations in endothelial cells and possibly a proxy of VEGF action. Therefore, it is possible that PRC2 regulation is dependent on SUZ12 and not EZH2. This is interesting because EZH2 is the major methyltransferase component for PRC2 and has been demonstrated to regulate miR-200b in neoplasia. EZH2 is often regarded as the most important component for PRC2 regulation, however in our study SUZ12 was shown to be important for miR-200b regulation. One possibility is that silencing of EZH2 in HG is insufficient to restore miR-200b levels to normal levels because SUZ12 is still able to occupy the miR-200b promoter region. Studies have shown that recruitment of PRC2 is dependent on SUZ12 (82,83,99). Furthermore, EZH1, a homolog of EZH2, also catalyzes for H3K27me3 and has been shown to compensate for EZH2-knockdown by replacing it in PRC2 complex (100,101). Thus, silencing of SUZ12 prevents methylation at the miR-200b promoter region by preventing overall recruitment of PRC2, while silencing of EZH2 may be compensated by EZH1. Although EZH1 was not examined in this investigation, it may explain why SUZ12 appears to be important in PRC2-mediated regulation of miR-200b. Additional experimentation is necessary to elucidate the interplay of these specific components of PRC2.

Finally, additional correlational evidence using other models, including animal models and other endothelial cell types, suggests the potential

importance of PRC2 in diabetic retinopathy. In retinal tissue from diabetic rats and mice EZH2, EED and SUZ12 expression was increased relative to non-diabetic control animals. Therefore, this mechanism may be relevant *in vivo* and requires further investigation to determine the therapeutic potential of inhibiting these targets to prevent the microvascular changes associated with diabetes. Furthermore, PRC2 components were elevated in HDMECs exposed to HG isolated from healthy individuals, as well as individuals with type 1 and type 2 diabetes. This further strengthens the mechanism that PRC2 components are elevated in HG in endothelial cells. Furthermore, the increase in HG observed in endothelial cells isolated from diabetic patients offers some interesting conclusions. These cells were isolated from patients who had diabetes for several years (ie. greater than 20 years). However, when treated in HG, PRC2 components were still overexpressed suggesting that hyperglycemia-induced upregulation does not become dampened. Thus, PRC2 and miR-200b may be important in other complications of diabetes.

Many different experiments were performed to elucidate this regulatory mechanism. One major limitation is that most of this work was done at the RNA/transcript level. Since this project was focused on determining expression changes at the level of the genome, RT-PCR was chosen as the major technique used to show changes. While expression at the RNA and protein levels are often parallel, replication of the changes at the protein level, such as by using Western Blotting or ELISA, would further strengthen the results. Furthermore, the knockdown efficiency between EZH2 and SUZ12 siRNA transfections differed

slightly, which may also explain why EZH2 silencing was ineffective in elevating miR-200b levels while SUZ12 silencing was effective. Finally, tube formation assay was used as a proxy of VEGF activity. Direct testing using ELISA or other techniques, such as a permeability assay, would further strengthen these results. None-the-less, the battery of experiments performed suggests increased PRC2 expression and activity at the miR-200b promoter region, providing rationale for why miR-200b becomes repressed in diabetes.

While other work in the field of epigenetics in diabetic complications has focused on either histone methylation or miRNAs, this project has shown how these epigenetic mechanisms work together to regulate VEGF. This project has demonstrated that PRC2 negatively regulates miR-200b and thus appears to be a good therapeutic target in diabetic retinopathy. Due to its nature as a multimeric enzyme complex, it can potentially be targeted with a small molecule. While much work is needed to design a chemical inhibitor that would selectively target this complex and be effective in being transported to the retina, an organ with its own selective permeability, this work has demonstrated another therapeutic strategy, which may show efficacy in the prevention of diabetic retinopathy. It is hoped that continued investigation into this complex will lead to a small molecule therapy for diabetic retinopathy that can be more accessible and cost effective in treating diabetic retinopathy, though such goals are far in the distance.

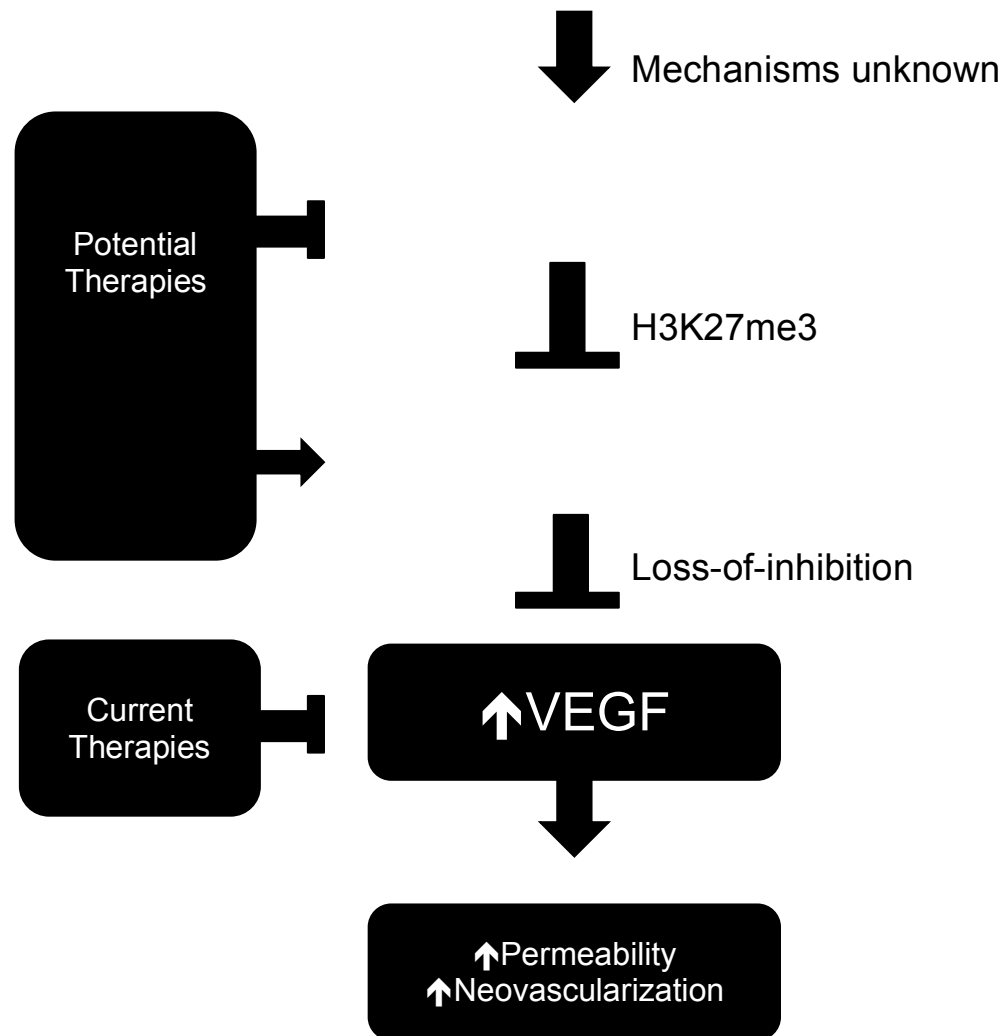
In summary, we show that PRC2 regulates miR-200b in retinal endothelial cells through H3K27me3 repression (Figure 4.1). This is one of the first

investigations to connect histone methylation and miRNA regulation in the context of diabetic retinopathy. Ultimately, this work builds on the characterization of miRNA our lab has studied by elucidating a new regulation mechanism. Investigating such mechanisms is important to further our understanding of the signaling events that occur in response to hyperglycemia in diabetic retinopathy, as well as developing novel treatment strategies to maintain the quality of life of patients with diabetes.

Figure 4.1

Summary diagram of key findings. Findings of this study demonstrate a regulatory relationship between PRC2 and miR-200b, of which SUZ12 appears to be the most important component in mediating this regulation (indicated by *). PRC2 is increased by high glucose, leading to repression of miR-200b and subsequently causing a loss-of-inhibition on VEGF levels. Altered VEGF production can lead to increased vascular permeability and neovascularization in diabetic retinopathy, demonstrating potential importance of PRC2 as a therapeutic strategy.

DIABETES



Future Directions

To further determine the efficacy of PRC2 inhibition in the prevention of microvascular changes in diabetic retinopathy, an *in vivo* model of diabetes would be suggested. Intraocular injection of diabetic rats with SUZ12 siRNA

appears to be the most effective in the study of this complex in diabetic retinopathy, based on the *in vitro* results. Furthermore, while the linear regulation of PRC2, miR-200b and VEGF was focused on in this project, PRC2 and miR-200b have been demonstrated in other studies to regulate genes of relevance in diabetic retinopathy. For example, PRC2 has been demonstrated to regulate VASH1, an anti-angiogenic factor. Inhibition of PRC2 may lead to increased VASH1, which may be desirable to prevent permeability changes and neovascularization associated with diabetic retinopathy (79,85). Also, miR-200b has been shown to regulate p300, a histone acetyltransferase associated with activation of matrix protein expression in diabetic retinopathy (53). Thus, while PRC2 was demonstrated to regulate miR-200b directly and VEGF indirectly in diabetic retinopathy, many potential interactions may be relevant. This highlights the importance of this complex in diabetic retinopathy as a therapeutic target. Additional experimentation in this area could further strengthen the potential therapeutic efficacy of PRC2 inhibition in diabetic retinopathy.

Finally PRC2 has numerous interactions with lncRNA, which opens up the possibility for future research into an extremely novel field. LncRNA (ie. HOTAIR) act as molecular scaffolds and may mediate other interactions with histone remodeling complexes (102). Furthermore, lncRNA (ie. ANRIL) act as recruitment flags for PRC2 to mediate specific association to genome regions (83). Investigations into these lncRNA in parallel with PRC2 may reveal a more complex interplay of epigenetic factors which together coordinate gene regulation.

Chapter 5: References

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Curriculum Vitae

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Education

2012 to present University of Western Ontario

- Master of Science Candidate
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2008-2012 University of Western Ontario

- Bachelor of Medical Sciences, Honors
Specialization in Pathology and Toxicology

Awards

2013 Dutkevich Memorial Foundation

- Travel Award in Pathology

2013-2014 Ontario Graduate Scholarship (OGS)

2012 Diabetes Research Day, London, ON

- Top Poster Presentation

2012 Western Undergraduate Research Forum, London, ON

- Top Poster Presentation

2012 Annual Pathology and Toxicology Research Day, London, ON

- Second Place for Outstanding Poster Presentation

2008-2012 University of Western Ontario

- Dean's Honor List

2008 University of Western Ontario

- Entrance Scholarship and Bursary

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Work Experience/Teaching Activities

2013 Graduate Teaching Assistant
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Published Papers/Manuscripts

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 miRNA-1 regulates endothelin-1 in diabetes. *Life Sci.* **98**,
 18-23 (2014). Published.
- 2013 Ruiz, M.A. & Chakrabarti, S. microRNAs: the underlying
 mediators of pathogenetic processes in vascular
 complications of diabetes. *Can. J. Diabetes.* **37**, 339-344
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- Feng, B., Ruiz, M.A. & Chakrabarti, S. Oxidative stress
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Presentations

- 2014 **Ruiz, M.A.** SUZ12 regulates glucose induced VEGF
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 endothelial cells. Annual Pathology Research Day,
 University of Western Ontario, London, ON. Platform
 Presentation.
- Ruiz, M.A.** Polycomb repressive complex 2 regulates
 glucose induced VEGF production through miR-200b
 regulation in retinal endothelial cells. London Health
 Research Day, London Health Sciences Centre, London,
 ON. Platform Presentation.
- 2013 **Ruiz, M.A.** Histone methyltransferase EZH2 regulates
 glucose induced VEGF production through H3K27
 methylation in retinal endothelial cells. Diabetes Research
 Day, Lawson Health Research Institute, London, ON.

Poster Presentation.

Ruiz M.A. Investigating a repressing histone methyltransferase EZH2 and its involvement in microRNA expression in endothelial cell models of diabetes. Annual Pathology Research Day, University of Western Ontario, London, ON. Poster Presentation.

Ruiz, M.A. Investigating a repressing histone methyltransferase EZH2 and its involvement in microRNA expression in endothelial cell models of diabetes. London Health Research Day, University of Western Ontario, London, ON. Poster Presentation.

2012

Ruiz, M.A. Investigating the involvement of EZH2 and H3K27 methylation in diabetic retinopathy Diabetes Research Day, Lawson Health Research Institute, London, ON. Poster Presentation.

Ruiz, M.A. Investigating the involvement of endothelial-derived miR-200b in diabetic nephropathy. Western Undergraduate Research Forum, University of Western Ontario, London, ON. Poster Presentation.

Ruiz, M.A. Investigating the involvement of endothelial-derived miR-200b in diabetic nephropathy." Pathology and Toxicology Research Day, University of Western Ontario, London, ON. Poster Presentation.

Abstracts

2013

Ruiz, M.A., Feng, B., Mortuza, R. & Chakrabarti, S. Histone methyltransferase EZH2 regulates glucose induced VEGF production through H3K27 methylation in retinal endothelial cells. 49th annual meeting of the European Association for the Study of Diabetes, Barcelona, Spain. Diabetologia (2013) 56:[Suppl1]S521. Poster Presentation.

Ruiz, M.A., Feng, B., Mortuza, R. & Chakrabarti, S. Histone methyltransferase EZH2 regulates glucose induced VEGF production through H3K27 methylation in

retinal endothelial cells. 73rd Scientific Sessions of the American Diabetes Association, Chicago, IL. Late Breaking Abstract 12-LB Diabetes(2013)62(suppl1A)LB4. Poster Presentation.